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THE ELECTRICAL STIMULATION OF MUSCLE, AS DEPENDENT UPON THE RELATIVE CONCENTRATION OF THE CALCIUM IONS.

By FRANK W. BANCROFT.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

Introduction. In a previous paper (1906)[1] I have shown that in paramecium the result of galvanic stimulation is very different according to the relative concentration of the Ca ions in the surrounding medium. In culture fluids, which contain considerable quantities of Ca, the constant current stimulates the infusorian at the cathode. In solutions containing less Ca the stimulation occurs at the anode; and in solutions containing still less Ca the current fails to stimulate at all. It was also found that all of these results could be explained in accordance with Loeb's theory of electrotonus (1905, 1907). On the basis of this theory I concluded that galvanic stimulation depends upon the production within that part of the paramecium stimulated of a certain definite value for the ratio $\frac{C_{Ca}}{C_S}$ (S being any salt producing an absence of galvanotropism). More recently I have made the observations on muscle given in this Paper, and these lead me to a more generalised statement on the relations of ions to galvanic stimulation, viz. that the effective galvanic stimulation of muscle must either consist in or be associated with a diminution in the ratio of the concentration of the Ca to the concentration of the Na ions[2], $\frac{C_{Ca}}{C_{Na}}$ which diminution must take place to at least a certain extent, and with at least a certain minimum

[1] For references see end of Paper.

[2] Since in this respect Mg acts like Ca and K acts like Na, the more accurate way to write this ratio would be $\frac{C_{Ca,\,Mg}}{C_{Na,\,K}}$. To avoid confusion the Mg and K and other similar ions have been left out of the ratio.

velocity, and which must be accompanied by at least a certain diminution in the absolute concentration of the Ca ions.

Methods. The frog's sartorius was used almost exclusively. *R. pipiens* was the species commonly employed, though a few individuals of *R. draytonii* were also experimented on. Both spring and summer frogs were used. They were usually not curarized, as the curare when administered was found to make no difference in the results. The muscles were immersed in a glass and paraffine trough (75 mm. × 25 mm. × 4 mm.) in the solution to be tested. Usually the muscle was neither stretched nor weighted. When it was desired to obtain graphic records of the contractions, the muscle was attached at one end to a loop of thread melted in the paraffine, and at the other to a thread which passed horizontally to an upright pin on a muscle lever. In all cases the muscle was kept in the solution during the whole course of the experiment, and (unless the contrary is stated) stimulated in the solution by constant currents conducted to the solution through non-polarizable electrodes. When graphic records of the contractions were obtained the thread to the muscle lever passed out of the solution at such a small angle that the end of the muscle was not lifted out of the solution.

The current used was a 110 volt direct current which was led through a water rheostat, milliammeter, pole-changer, and Porter's non-polarizable boot-electrodes to the trough. As it was frequently necessary to leave currents of eight or more milliamperes flowing through the trough for considerable periods the $ZnSO_4$ in the electrodes was replaced by NaCl or the same solution that was being used in the trough. In this way the contamination by SO_4 ions was prevented and that by the Zn ions so delayed that no trouble was ever experienced from that cause. The strength of the current varied from 1 to 20 milliamperes; 8 ma. was the strength most often employed. As the cross section of the trough used was 100 sq. mm. the density of the current in δ is obtained by multiplying its strength in milliamperes by 10. The chemicals were all of Kahlbaum's best grade.

Experimental results and their explanation.

It has often been insisted upon that in electrical stimulation we should distinguish clearly between the effects of the rapid change in the intensity of the current in making and breaking, and the effects produced during the flow of the current. In the present investigations

the three well-known effects of the galvanic current could usually, though not always, be distinguished. These are:—

(1) The muscle twitch at make and break.

(2) The lasting contraction (*Dauercontraction*) which appears at make and continues for a while during the flow of the current. If the current is broken the lasting contraction immediately disappears. If the current is not broken it also ultimately disappears, but much more slowly and gradually. Upon breaking, a similar lasting contraction may appear, which immediately relaxes if the current is made again, but which gradually disappears if the current is kept broken.

(3) The fibrillary or rhythmic twitchings, similar to those which occur when the sartorius is placed in a sodium chloride or citrate solution. These vary more in their relation to the current than the two previous phenomena, but still exhibit sufficient regularity to show that they obey a different law from the make and break twitch.

These three different effects of the current probably depend upon the presence of what we may call three different "contractile substances" in the muscle without attempting to decide whether these substances are localized in different kinds of fibres, as is supposed by Grützner and Zennek, or in the fibrillæ and sarcoplasm as is held by Botazzi, or in some other way.

Results with the graphic method and NaCl muscles[1].

These results are concerned mainly with the make and break twitch, and to a less extent with the lasting contraction. The fibrillary twitchings can be studied much better by simple observations in the trough.

In repeating these experiments it should be kept in mind that the details have been chosen so as to obtain the simplest and clearest results possible, and if any of these details are much changed, the same results will probably not be obtained. Thus the current was chosen rather strong (8 ma.) so as not to prolong the experiment. In some cases in order to obtain a break contraction the current was kept flowing half a minute so as to get a strong effect. In other portions of the same experiment where the influence of the diffusion on the break contractions was being examined, the current was kept flowing

[1] Professor W. E. Garrey informs me that he has independently discovered the effect of the relative concentration of Ca on the make and break twitch, and has used them for laboratory experiments for some time.

only for a few seconds for each break contraction, so as to complicate the effects of diffusion as little as possible. The invariable result in my experiments with muscles in NaCl was that the make contractions failed before the break contractions. If, however, the testing current had been made only for a few seconds instead of half a minute or a minute, it might easily have happened that the break contractions failed before the make contractions. The details of each experiment are given in the explanation of the figures. But while I do not know just what will happen if the strength or duration of the current or the concentrations of the solutions are changed much from the values which I have used, the general results are so clear that I feel convinced that they will prove essentially the same provided that the comparative method is strictly adhered to.

When a sartorius muscle was put in the trough in m/8 NaCl solution and every few minutes a constant current of 8 milliamperes was allowed to flow through the trough for a half or a whole minute, it was seen that at first the muscle contracted both when the current was made and when it was broken, and that the contraction at make was the more vigorous (Fig. 1). As the muscle remained in the solution it soon became injured and responded to the same stimuli with weaker contractions, but in each pair of contractions the make twitch was always the stronger. After from about 20 to 30 minutes however, the make twitch decreased in height much more rapidly than the break twitch, so that in each pair of contractions the make twitch became weaker and weaker, and finally disappeared entirely. At this time the only response to the current was a break twitch which was very much reduced in height (Fig. 1).

The explanation of this result is as follows: since galvanic stimulation depends upon the decrease of the $\frac{C_{Ca}}{C_{Na}}$ ratio, when the muscle is placed in the NaCl solution, this ratio soon becomes decreased to such an extent that it is impossible for the current to decrease the Ca ions much more at the cathode, and hence the make stimulation, which takes place at the cathode, does not occur. But the sojourn in the NaCl has diminished the ratio in all parts of the muscle so that, while formerly a current lasting for one minute could bring about a considerable increase in the ratio at the anode, and thus cause a strong contraction upon break, now the same current lasting for the same length of time will produce a much smaller increase in the ratio at the anode. Now the rapidity of the diffusion of the ions away from the anode depends upon

the fall in the concentration of these ions in the direction away from the anode. Consequently when the ratio has only been slightly increased at the anode it can only be slightly decreased when the current is broken. This decrease will also take place comparatively slowly, and hence the stimulus and the contraction when the current is broken should be much smaller than when the muscle was first put in the

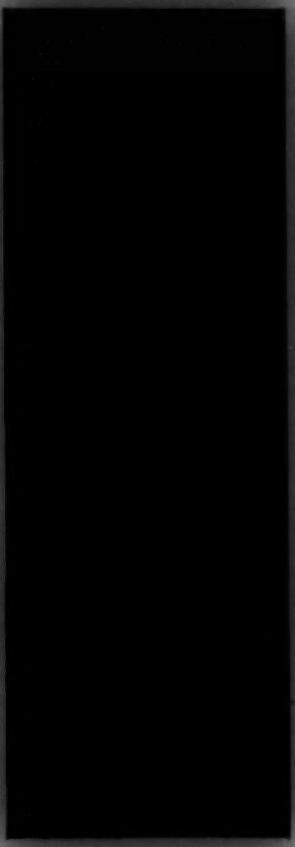

Fig. 1. Contractions of a frog's sartorius in m/8 NaCl solution reduced ½. Read from right to left. Current is 8 ma. Signal magnet (lower line) is on a separate circuit. The down stroke of the magnet records the make of the current. First contraction recorded after the muscle had been in the solution 20 minutes. For the first 30 minutes of the record the current was made for 1 minute every 5 minutes. Make contractions have entirely disappeared, leaving only the much reduced break contractions. Now a current passing in the same direction as before is kept flowing continuously except for a rapid break and make every half minute. The break contractions increase in height with the flow of the current. At * the current is reversed and kept flowing continuously in the new direction except for a rapid break and make every half minute as before. After a period of quiescence the break contractions begin again and increase in size. At *B* the current is interrupted and made only for 5 second periods every half minute. The contractions are as before, all break contractions, and decrease gradually in size until they disappear.

NaCl solution. An examination of Fig. 1 shows that these contractions are as the theory requires.

It might be contended, however, that the weakness of these contractions has nothing to do with the $\frac{C_{Ca}}{C_{Na}}$ ratio, but is due solely to the injury of the muscle in the solution. If, on the other hand, the theory is correct, then, since the longer the current flows the greater will be the increase of the ratio at the anode, it should be possible to obtain stronger break contractions by allowing the current to flow in the same direction for a longer time and then breaking it; and this is actually found to be the fact. These results are also shown in Fig. 1, in which after the make contractions had entirely disappeared, and the break contractions had been very much reduced, the current was made and kept flowing for 9½ minutes, being broken and made again rapidly every half minute. All the contractions are break contractions, and the record shows that with the flow of the current they increase rapidly in height, until quite vigorous contractions are obtained.

It should also be noted that the curve obtained by joining the tops of the lines indicating each contraction, rises at first in approximately a straight line, then has a point of inflection, and runs nearly parallel to the abscissa. This is exactly the form of the curve required by the theory, for at first the concentration of the ions at the anode would be nearly directly proportional to the time that the current has flowed, while later when the current has been flowing longer the rapidity of the diffusion away from the anode would increase *pari passu* with the concentration of the ions there by the current, until finally the two opposing forces would balance each other.

Another objection that might be advanced against this interpretation is that the increased strength of the contractions is due merely to some unknown beneficial effect of the current and is not dependent upon the accumulation of the ions at the anode. The reversal of the current entirely does away with this objection. The result of the reversal is also illustrated in Fig. 1. When the current is reversed at *, and kept flowing in the opposite direction, being broken and rapidly made again every half minute, it is seen that for 5 minutes there is no response to either make or break. Then the break contractions begin to appear, and gradually become vigorous again. The explanation of this fact is evident. While the current is flowing in one direction certain ions accumulate at the anode and make stimulation possible. When the current is reversed, the old cathode becomes the new anode,

and it takes time for enough of the required ions to accumulate at the new anode to make stimulation possible.

When the current is broken after it has been flowing for some time in one direction the ions which have accumulated at the anode should diffuse away again, and thus gradually the strength of the contractions should diminish. This result is also illustrated in Fig. 1. At "break" the current was broken, and after that made only for 5 second periods every 30 seconds. All of the contractions were break contractions, and they progressively diminished in strength until, 4 minutes after the current was broken, no contractions at all were obtained.

Fig. 2. Contractions of completely curarized frog's sartorius in m/8 NaCl solution reduced ¼. Arrangement as in Fig. 1. The first contraction is after the muscle had been 16 minutes in the NaCl. After the first stimulation the ascending current is made for half a minute every 2 minutes. At *m* the ascending current is made and kept flowing except for a rapid break and make every half minute. The contractions are all break contractions. At "Reverse" the current was reversed and broken again in a few seconds. After this the current in the reversed direction was alternately made and broken every 10 seconds for 60 seconds. During this interval the muscle contracted only at the make of the current; but after 60 seconds, during which the current had been flowing 30 seconds, the make contractions failed. The descending current was now kept flowing continuously except for a rapid break and make every half minute. During this period there are no contractions for a few minutes, but after that they begin again, but now all the contractions are break contractions. At *R'* the current was first broken, then the pole changer reversed; but the circuit remained open except for a rapid make and break every 15 seconds. The contractions, which are now all make contractions, gradually diminish in size and disappear.

One further point needs elucidation. When a current that has been flowing for some time in one direction is reversed, the old anode at which the $\frac{C_{Ca}}{C_{Na}}$ ratio has been increased now becomes the new cathode; and since the ratio has been increased there, it ought to be possible now to get make contractions at the cathode, for now the current can

reduce the ratio in that region. These results were also obtained, and are illustrated in Fig. 2. After each of the two reversals indicated there, a diminishing series of contractions was obtained, but while all the contractions before the reversal were break contractions, after the reversal they were all make contractions. After the first reversal in Fig. 2 the current was continued flowing in the reversed direction for 10 seconds, and then broken for 10 seconds. As was to be expected these make contractions soon failed, for the ions which made the galvanic stimulation possible were rapidly removed from the cathode by the current and by diffusion. At the second reversal in Fig. 2 the current was first broken, then the pole-changer reversed. The current was not kept flowing continuously, but was made and broken rapidly every 15 seconds. By this procedure the ions are removed from the region of the new cathode almost solely by diffusion, and it is evident from the record that diffusion alone takes very much longer to remove the ions from the region of the cathode than is required by the forces of diffusion and the current acting together.

These considerations also explain why the contraction obtained when the current is reversed is always considerably higher than the break contractions preceding it; for at the reversal there is a break stimulus at the old anode followed very rapidly by a make stimulus at the new cathode.

These phenomena are essentially the same as the well-known facts of the voltaic alternatives, in which the constant current produces in muscles, which have not been subjected to a NaCl solution, a condition in which they respond more strongly to the break of the current, and to the make of the current in the reverse direction, even when they entirely fail to respond to the make of the current flowing in the original direction (Hermann's *Handbuch*, I., Part 1, p. 95; Burdon Sanderson, 1900, p. 430; Biedermann, 1895, pp. 191—192; 1879, p. 292). These voltaic alternatives have usually been attributed to a local fatigue of the muscle produced by polarization.

Considering, then, the results obtained with the muscle in NaCl, it is evident that the facts correspond with the theory even so far as the smallest details are concerned, and it seems to me that there can be no doubt that even if this theory is not accepted, it has been rendered very probable that: Galvanic stimulation depends upon the presence in sufficient concentration of certain anions. According to the theory here advocated these anions are, under these conditions, mainly the Cl ions, and they act, not directly but indirectly, by increasing the

concentration of the free Ca ions through the formation of $CaCl_2$. In this way the value of the ratio will be increased, and thus the opportunity given for its decrease and the accompanying galvanic stimulation at the break of the original current, and at the make of the oppositely directed current.

Results with the Graphic Method and $NaCl + CaCl_2$.

When a sartorius muscle was placed in a mixture of about 98 volumes m/8 NaCl + 2 volumes m/8 $CaCl_2$, and every few minutes a constant current of 8 milliamperes passed through it for half a minute, it behaved entirely differently from a muscle in pure NaCl solution (Fig. 3). These proportions are approximately those in which the Na and Ca exist in the blood, and as would be expected this solution modifies the normal behaviour of the muscle but slightly. The contractions maintained more nearly their original height; but the most important result is that throughout the whole experiment the make and break contractions were of about the same height and varied together. Even when the current was made and kept flowing in one direction for a while there was no change to speak of and the make and break contractions remained similar to each other.

When, however, the proportion of Ca was made considerably greater than what exists in the blood, by using a mixture of 90 volumes m/8 NaCl + 10 volumes $CaCl_2$, then, as the time went on, the make contractions maintained their original vigour, while the break contractions decreased in size and finally disappeared (Fig. 4). Here also a current kept flowing continuously in one direction did not materially change things.

As was indicated in the introduction, the results obtained with an excess of Ca become intelligible when we consider the relative amounts of organic anions and of Ca which are present within the muscle tissue. Since there is now such an excess of Ca present, the absolute amounts of ions which are required to diminish or to increase the $\frac{C_{Ca}}{C_{Na}}$ ratio are much larger than they are in the normal muscle. According to the theory the ratio is reduced at the cathode by the formation of slightly soluble and slightly dissociated Ca compounds, so the amount by which this ratio can be reduced is limited by the amount of these organic anions which are available. At the anode where the ratio is increased by the current, the Ca ions for increasing it are liberated from their

compounds by the arriving Cl ions, and hence must come from the muscle tissue, and the amount by which the ratio can be raised is limited by the amount of Ca available. Now as the excess of the Ca in the solution is increased, the absolute amounts of the organic anions

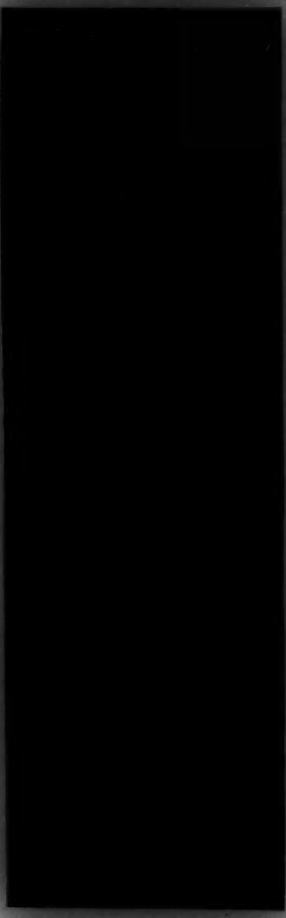

Fig. 3. Contractions of frog's sartorius in 99 c.c. m/8 NaCl + 2 c.c. m/8 $CaCl_2$, reduced ½. Current is 8 ma., descending. Rise of signal magnet indicates the make of the current. The first stimulation is after the muscle has been 5 minutes in the solution. After this, the current was made for half minute intervals every 4 minutes. At "make," when the muscle had been in the solution 65 minutes, the current was kept flowing continuously except for a break of 15 seconds every 2 minutes. *S* indicates in each case a series of spontaneous rhythmical contractions.

and of Ca required for stimulation at cathode and anode respectively must also increase. Since we have every reason to suppose that there is a much greater supply of the organic than of the Ca ions it is natural that a point should be reached at which the supply of the Ca ions fails and the break stimulation becomes impossible.

Fig. 4. Contractions of sartorius in 90 c.c. NaCl + 10 c.c. $CaCl_2$, reduced ½. Fall of signal magnet indicates make of current. Current is 8 ma., descending. First stimulation is after the muscle has been in the solution 9 minutes. After that the current was made for 30 seconds (once 40) at 6 or 5 minute intervals. At "make" the current was made continuously except for a break of 15 seconds every 2 minutes. The effect of the solution was to decrease and finally to suppress the break contractions.

This I consider the main cause for the failure of the break contractions. But there is another which probably contributes to this result. We have seen that at the cathode the $\frac{C_{Ca}}{C_{Na}}$ ratio is reduced by the direct migration of the ions in the electrical field, while at the anode the ratio is reduced after the break by the diffusion away of the ions. The results illustrated in Figs. 1 and 2 show that under the conditions here employed the current is much more effective in reducing the ratio than the forces of diffusion. Thus in the experiment illustrated in Fig. 1, after the current had been broken at the end of the experiment it took 3½ minutes for the ions to diffuse away from the anode to a sufficient extent to prevent stimulation. If the current had been merely broken at the time when it was reversed the power to contract would have persisted for at least the same length of time. But we see that it disappeared within half a minute when the current was reversed. Thus the current and diffusion working together accomplished the same result in less than ⅛th of the time that the

diffusion alone did. Similar results are shown in Fig. 2. Consequently it is clear that when the conditions are made less favourable for stimulation by increasing the Ca it should be the anodal break stimulus, which results from the diffusion of the ions, that should fail first[1].

Results by the graphic method with K, Mg, and Ba.

The chlorides of K, Mg, and Ba, were in all cases mixed with NaCl, since in their pure solutions they injured the muscle too rapidly to make it profitable to try for graphic records.

In mixtures of NaCl + KCl the make contraction soon failed, just as it did in pure NaCl. About the only difference to be seen was that when the KCl was present the muscle suffered more rapidly.

Mixtures of NaCl + $MgCl_2$ acted much like mixtures of NaCl + $CaCl_2$, but strange to say 98 vol. m/8 NaCl + 2 vol. $MgCl_2$ acted less strongly in suppressing the break contractions than the corresponding mixture of NaCl + $CaCl_2$, while both the mixtures of 95 vol. NaCl + 5 vol. $MgCl_2$ and of 90 vol. NaCl + 10 vol. $MgCl_2$ acted much more strongly than the corresponding mixture of NaCl + $CaCl_2$. The number of experiments made was small, and more data would probably clear up the discrepancies.

Mixtures of NaCl + $BaCl_2$ acted in general like mixtures of NaCl + $CaCl_2$ so far as the make and break twitches were concerned. They brought about the disappearance of the break contractions even more rapidly than the corresponding mixtures of NaCl + $CaCl_2$.

It was in their effect on the lasting contraction that solutions containing $BaCl_2$ had their most pronounced effect. In pure NaCl solutions and in mixtures of NaCl + KCl this lasting contraction was hardly to be noticed at all. In mixtures containing $CaCl_2$ or $MgCl_2$ it was noticeable but small. In mixtures containing $BaCl_2$, however, it was always a pronounced feature of the reaction. When the muscle had been in a solution containing $BaCl_2$ but a short time it gave pronounced make twitches and much smaller lasting contractions. After it had been in the solution 10 or 15 minutes the make twitches had usually decreased in height until they were no higher than the lasting contractions. The curves now presented the double peak characteristic of veratrin muscles. Soon the twitch disappeared entirely, leaving only the slow lasting contraction which persisted for an hour or

[1] The same considerations also explain why it is that normally the make of a galvanic current is a more effective stimulus than its break.

so longer. It was thus possible in this case to study the "substance" responsible for the lasting contraction unencumbered by any other reactions. Under these circumstances break contractions could only be obtained after the current had been flowing several minutes, while make contractions could always be promptly obtained. After the current had been flowing for several minutes in one direction the make of the reversed current always produced a very much stronger contraction than usual, quite as high as a vigorous twitch but of the slow long drawn out character of the lasting contraction. It was thus evident that to a certain extent the lasting contraction obeys the same law as the make and break twitch. There are some differences however, which have not as yet been fully worked out, but which seem to indicate that the lasting contraction "substance" responds to the galvanic current in a way intermediate between that shown by paramecium and by the make and break twitches of the sartorius.

Biedermann's results with the lasting contraction (1890, pp. 264—271; 1895, p. 179) point strongly in this direction. He found that with weak currents the make lasting contraction was confined to the cathode, while with stronger ones it became anodal in its distribution. This is the sort of thing that would be expected if the stimulation depended, as in paramecium, upon a certain definite value of the $\frac{C_{Ca}}{C_{Na}}$ ratio. For, if the ratio is normally too high, it would be reduced to its stimulating value only at the cathode. As the current continues flowing, however, especially if it be strong, the ratio should soon be reduced too low at the cathode, and the stimulating value of the ratio should only exist in some place in the intrapolar region. Finally, as the current lasts still longer, or is still stronger, this place of stimulation should approach the anode, and the response observed by Biedermann should result.

Results from the simple observation of unweighted muscles.

Most of the experiments were performed in this way without any attempt at graphic registration. The muscle was laid in the trough, parallel to the current lines, without any weighting or stretching and observed both without and with a slight magnification. In some cases, where the muscle curled up too much, a cover-glass was laid over it to keep it flat; but this was soon abandoned as it prevented or delayed the characteristic effect of the chemical, presumably by preventing

diffusion. This method is a much more delicate one than the graphic method and its results should be always used to control those obtained by the graphic method.

The solutions used were in general m/8, except for the acids which were m/10. The following substances were used either in pure solutions or combined in various ways:

NaCl	NH_4NO_3
Na_2 citrate	$CaCl_2$
Na butyrate	$BaCl_2$
NaF	$MgCl_2$
Na_2 oxalate	Butyric acid
Na_2SO_4	Citric acid
K_2 oxalate	Oxalic acid
LiCl	Urea

According to the effects produced these solutions may be divided into three classes:

(1) Those causing break contraction to persist longer than make contraction.

(2) Those causing make contraction to persist longer than break contraction.

(3) Those causing both make and break contractions to disappear together.

To class 1 belong all the Na salts and K_2 oxalate. Urea is the only representative of class 3. All the other solutions belong to class 2.

The salts of class 1. These all act like NaCl, but this last salt acts less rapidly than any of the others. This is of course because the other salts diminish the numerator of the $\frac{C_{Ca}}{C_{Na}}$ ratio by decreasing concentration of the Ca ions, as well as increasing the denominator. Thus they decrease the value of the ratio more rapidly than NaCl, which will mainly affect the denominator. An experiment with NaF, which was probably the most rapidly acting salt, may be cited as representing the kind of results obtained.

Fresh sartorius put in trough in m/8 NaF and tested at the times indicated with a 4 ma. current.

1½ mins. Good make twitch and break twitch, both extending over the whole muscle.

3 mins. No make twitch at all. Good break twitch.

7 mins. Absolutely no make twitch. Good break twitch.

12 mins. Very slight make twitch at cathode with descending current. With ascending

current there is a slight motion at make which I cannot localize. Good break twitch with both currents.

17 mins. Ascending current: very slight make twitch; fair break twitch.

18 mins. Descending current: one slight make twitch at cathode which could not be repeated. Break twitch obtained with every stimulus.

19 mins. Ascending current: same as above.

20 mins. Descending current: same.

22 mins. Descending current: no make twitch; slight break twitch.

23 mins. Ascending current: very slight make twitch and break twitch. Muscle hardly contracts at all.

This is a fairly typical experiment in which the make twitch persisted in greater force than usual. In many experiments with NaF, Na_2 oxalate, and Na_3 citrate the make twitch failed after 10 or 15 minutes and did not reappear again. When it did reappear, however, or when it persisted to a certain extent throughout the whole experiment, it was always very much weaker than the break twitch. The most characteristic feature of this persisting make twitch was that it could only be obtained after a rest. The height of the twitch was greater the longer the rest, but in such cases the available material was entirely exhausted by the single make contraction, and a second contraction could not be obtained until the muscle had rested. Nothing of this nature was ever noticed with the break contractions, except possibly in the case of the lasting contractions in NaCl + $BaCl_2$ already mentioned. The break contractions could always be repeated indefinitely provided the current was strong enough and lasted long enough.

The cause of this characteristic regeneration of power to respond to a make stimulus would appear to lie in a slow transformation of unavailable Ca into some available form. If, after the contraction, the Ca were left in the ionic state, then, on account of its low concentration in the surrounding fluids it would soon become lost to the contractile substance, and the Ca for the next contraction would have to come from the slow transformation of some compound in which it was present in an unavailable form. Martin (1904) for the terrapin heart, and Guenther (1905, p. 93) for the sartorius of the frog have also discovered phenomena which they interpret as the transformation of the Ca from some unavailable into some available form[1].

[1] It is perhaps worth while calling attention to the fact that the period required for these muscles to recover their power to respond to the make of the galvanic current may be considered as a refractory period. It looks also as if the sartorius under these conditions obeys the law of all or none, though that question was not specially investigated. It is to be remembered however that according to Keith Lucas (*Journ. Physiol.* XXXVIII., 1909) the normal sartorius obeys the "all or none" law.

As the muscle remained in the solutions of class 1 the strength of the break contractions also decreased, though not so rapidly as the make contraction. At first a break contraction was usually obtained with a current of 2 ma. lasting one second or less. As the muscle remained longer in the solution either the strength or the duration of the current or both had to be increased in order to get a break contraction. I attempted to discover some quantitative relation between the strength of the current and the length of time it had to flow in order to produce a break contraction. But, on account of the variability of the results, I obtained little of value. All that can be said is that the weaker the current the longer it has to flow, and that the longer the muscle stays in the solution the stronger is the current which will not produce any contraction, even after a duration of 2 or 3 minutes.

The processes which take place when a muscle is placed in either a solution of class 1 or of $CaCl_2$ or $MgCl_2$ are of a thoroughly reversible nature, provided of course that the solution is not allowed to act too long. If a muscle has been left in one of the solutions of class 1 until the make twitch has entirely disappeared; this twitch can easily be made to reappear again by adding a little Ca. If still more Ca is added the break twitch in its turn fails, leaving only the make twitch. The reverse process can be accomplished just as easily.

Nothing has been seen in all the experiments which would justify the conclusion that it is possible to change the pole at which the make twitch and break twitch arises. In view of results on paramecium (1906) to be considered in greater detail in connection with the rhythmical twitchings, where it was found possible to change the pole at which the galvanic current stimulates, a similar reversal of Pflüger's law was anticipated here. For the lasting contraction such a reversal seemed to appear in a few cases in $NaCl + BaCl_2$, and for the rhythmic fibrillary twitchings a reversal is usually obtained, as will be described later. But for the make and break twitch no indications of such a reversal were seen. When the muscle was fresh these twitches were very rapid and extended to all parts of the muscle, so that a simple inspection could tell nothing of the pole at which the contraction originated. But when the muscle had been in the solution for a while the twitches became local, and then it was always seen that, no matter what the nature of the solution, the make twitch always occurred at the cathode, and the break twitch at the anode.

One other phenomenon may be mentioned. On one occasion a muscle was put in 10 vol. m/10 K_2 oxalate + 90 vol. m/8 Na_2 oxalate, and after remaining in the solution 16 minutes an ascending current of 8 ma. was passed through it for 15 minutes. It had then developed such a hypersensitiveness that touching the surface of the solution in the vicinity of the muscle with a glass rod or the removal of the rod from the solution even at the distance of a cm. from the muscle would stimulate it to contract. In spite of several attempts this result was never obtained a second time.

The chemicals of class 2 comprise:

$CaCl_2$	LiCl
$MgCl_2$	NH_4NO_3
$BaCl_2$	Butyric acid
	Citric acid
	Oxalic acid

In solutions of all these substances only make contractions were obtained, or the make contractions persisted after the break contractions had failed. But in some cases it was not clear what the significance of this result was. The effect of the first three chemicals was tested with pure solutions and also in combination with NaCl. This latter procedure showed clearly that these salts antagonized NaCl and thus certainly belong to class 2. For the other substances, however, the combination with NaCl was not tested, so that it is possible that their action may be due to a general injury of the muscle rather than to any specific action on the make or break contraction.

Pure m/8 $CaCl_2$ solution injured the muscle so much that no contractions could be obtained as long as the muscle was in the trough. But when the density of the current was increased by removing the muscle from the fluid and suspending it in the air between the electrodes, a current of 3·5 ma. sufficed to produce a slow contraction upon make which persisted during the flow of the current.

Pure m/8 $MgCl_2$ solution caused the break twitch to disappear in about 2 minutes. A slow make contraction which may have been a lasting contraction could be obtained for an hour longer.

Pure m/8 $BaCl_2$ solution acts similarly, but not so strongly. In this solution the break twitch disappeared in about 3 minutes. The make twitch persisted considerably longer, and the make lasting contraction could be obtained even after an hour in the solution.

Mixtures of all these three salts with NaCl showed that they antagonized the action of the NaCl solution in the same way observed with the graphic method.

Concerning the rest of the substances in class 2 little need be said. In these solutions the break twitch failed in from one to 12 minutes.

But as in some cases all contraction stopped a little later the interpretation of the results is somewhat doubtful.

In urea no contractions at all could be obtained after 25 minutes with a 2 ma. current; but as long as any contraction at all could be obtained the muscle contracted at both make and break.

Rhythmic fibrillary twitchings.

The effect of the current on the fibrillary twitchings is similar to that which I have already described for paramecium (1906).

Guenther (1905, p. 103) adopted the view that in the sartorius two contractile substances are present. He came to the conclusion that it is the same substance which gives the rapid make and break twitches as well as the rhythmical fibrillary contractions. My results on the other hand lead to the conclusion that the "substance" which is responsible for the rhythmic fibrillary twitchings is very different from the "substance" which produces the make and break contractions, and behaves towards the current in essentially the same way as paramecium does.

When a sartorius muscle is placed in any one of the solutions of class 1 it responds promptly with a series of rhythmic twitches. These are liable to be very violent at first, and so may not be affected much by the current. But after a few minutes they become more quiet, and if the current is made then it is usually seen that these twitchings become stronger in the anodal and weaker in the cathodal region. When the muscle has lain a still longer time in the solution without having been subjected to the current, the twitchings stop altogether. If now the current is made, the twitchings begin again in about a minute. They may be limited to the anodal region from the first, or they may begin in the centre or all over the muscle, and later become localized at the anode. If the current is kept flowing still longer, for about four or five minutes altogether, then the twitchings stop again. If the current is now stopped, the twitchings may reappear again in the region which was previously the anode. If after the current has been stopped it is rapidly started again in the opposite direction, then the twitchings may first appear at the cathode (which was the anode of the previous current) but gradually disappear from that region and become localized at the new anode. There are occasional exceptions, but in the great majority of cases the behaviour is as stated.

All of these facts can be easily explained in the same way in which

the galvanic stimulation of paramecium was explained. We must suppose that normally the $\frac{C_{Ca}}{C_{Na}}$ ratio is too high to make the fibrillary twitchings possible. When however this ratio is decreased by immersion in an appropriate solution the twitchings begin. After a while they stop because the ratio has become reduced below the proper value, which we may call the stimulating value. If now just before or just after they have stopped, a current is made through the muscle the ratio will be decreased in the cathodal region and increased in the anodal region, thus increasing or starting the contractions in this region. If the current is kept going the twitchings will continue at the anode for a while, but soon the ratio will be increased above the stimulating value and the twitchings will soon stop again. If now the current is stopped the ions that have been responsible for increasing the $\frac{C_{Ca}}{C_{Na}}$ ratio will diffuse away from the anode, the stimulating value of the ratio will be reached, and the twitchings will continue until in the process of diffusion the ratio again becomes too low. If after the current has been broken it is rapidly made again in the reverse direction the aggregation of the anions will now be at the new cathode, where the ratio will be rapidly diminished by both the current and diffusion to the stimulating value, and the twitchings will continue there for a while until the value of the ratio falls too low at the cathode, and reaches the stimulating value at the anode where the twitchings will then reappear.

In this way then all the facts of the fibrillary twitchings of the sartorius may be explained, and it must be concluded that there is in the sartorius another "contractile substance" in which stimulation is associated with a certain definite value for the $\frac{C_{Ca}}{C_{Na}}$ ratio, and not as in the case of the make and break contractions with the decrease of that ratio. Since this first "substance" behaves in essentially the same way as does the ciliary apparatus of paramecium it is permissible to assume that it is less differentiated and specialized than the make and break twitch substance. In the nerve which is still more specialized than the muscle it is probable that galvanic stimulation will follow a law somewhat similar to that for the specialized substance of muscle; but the experiments so far done on nerve seem to show that the conditions there are still more complex and less amenable to experimental control.

It must be confessed that in the case of the fibrillary twitchings I

have investigated only half the problem, and have not attempted to get these twitchings by means of the current when the $\frac{C_{Ca}}{C_{Na}}$ ratio is higher than the stimulating value; that is when the muscle is in approximately its normal condition. If this theory is true it ought to be possible to get such twitchings at the cathode in muscles which are normal, or have been only slightly subjected to solutions tending to diminish the relative concentration of their Ca ions. I have not as yet made any such experiments.

Enough has been done, however, to show that there are two types of response to the galvanic current, which are probably only the extremes of a long series of reactions: one in which it is not possible to reverse the poles at which stimulation takes place and the other in which this is possible.

Discussion of results.

Besides furnishing an explanation for the facts already mentioned the theory here advanced helps us to understand the fundamental facts of galvanic stimulation and many other facts contained in the extensive literature of electrophysiology. A few points only need be mentioned here.

Pflüger found at the cathode during the flow of the current a condition of increased excitability, and at the anode a condition of decreased excitability. Stimulation at make he considered to be due to the establishment of this condition of increased excitability, and stimulation at break he thought was due to the disappearance of the anelectrotonic decreased excitability. Loeb supposed that the increased excitability was caused by a diminution of the Ca ions at the cathode, and the decreased excitability by the increase of Ca ions at the anode. My theory of stimulation holds that the make stimulus is associated with a diminution in the relative concentration of the Ca ions at the cathode, and the break stimulus is associated with a similar decrease in the relative concentration of the Ca ions occurring at the anode after the break. This parallel is so exact, that, given Pflüger's conclusions, the present theory of stimulation might have been deduced directly from Loeb's theory of electrotonus, though as a matter of fact it was not so deduced.

The lack of stimulation during the flow of the current is one of the most difficult features of galvanic stimulation to explain. To

be sure it does not hold at all for galvanic stimulation in the case of paramecium or the fibrillary twitchings, and only to a limited extent in the case of the lasting contractions. But for the make and break twitches, which are by far the most prominent feature of the contraction of the sartorius, it holds very well. The explanation of this phenomenon, as indicated in the introduction, is that during the flow of the current the $\frac{C_{Ca}}{C_{Na}}$ ratio is nowhere decreased with sufficient rapidity. But why, if at make the ratio is decreased at the physiological cathode, that is, at the extreme end of each muscle fibre, does it not in the next interval of time undergo a similar decrease in that section of each muscle fibre immediately adjacent to the cathode?

In the first place, in considering this question we must realise the exceedingly small distance that the ions have to travel to cause stimulation. If it is assumed that Cl ions are the ones whose migration brings about stimulation, and that the portion of the muscle where stimulation takes place conducts the current like an m/8 NaCl solution, then taking the current as 8 ma. and the latent period as ·01 sec. (Tigerstedt, 1885, p. 200–) and the cross section of the trough 1 sq. cm., then the distance that the Cl ions would travel during the latent period can be figured out according to the method given in Le Blanc (1907, p. 120). This distance, which is of course the distance which the ions must travel in order to bring about stimulation, is ·0005 mm. or half a micron. It indicates only the average distance that the Cl ions will migrate.

Let us now compare the changes which occur at the extreme cathode during stimulation with those occurring in the next interval of time during which stimulation does not occur. The current enters and leaves each muscle fibre through the periphery, which is covered with membranes that are relatively impermeable to ions. Therefore we are justified in supposing that at the cathode either none or but relatively few Cl ions will enter the muscle through these membranes[1]. Consequently after the Cl ions have migrated away from the cathode under the influence of the current there will be but relatively few Cl ions left. Some Cl ions will be left, because all of the ions will not have moved with the same velocity and other Cl ions will have been formed by dissociation. During the next time interval during which stimulation does not take place, the Cl ions will be removed from the next section of the

[1] Ions of some kind must of course pass through the membrane to conduct the current.

muscle fibre in just the same way and to the same extent to which they have just been removed from the cathode section. But there is now no membrane of any kind between this second section and the cathode section, consequently the Cl ions which have been left behind in the cathode section, together with any Cl ions which may have migrated through the surface membranes, will pass into the second section. Thus the concentration of the Cl ions, and consequently of the Ca ions, will not be diminished as much in this second section of the muscle as in the cathode section, and in this way the absence of stimulation is explained[1].

The fact that ordinarily the make of a galvanic current is a more effective stimulus than its break receives a very natural explanation by means of this theory. The facts of the voltaic alternatives in normal muscle, and the similar results already described for muscle in NaCl, show that, no matter whether this theory is correct or not, the flow of the constant current gradually brings about at the anode the conditions required for both make and break stimulation at that particular part of the muscle. Now these conditions which make stimulation possible must depend upon certain changes in the concentration of the ions. These changes must be opposed by the tendency of the ions to become equally distributed throughout the muscle fibre by means of diffusion; and the fact that the changes in concentration occur shows that, at first at any rate, the current is stronger than the forces of diffusion tending to equalise the distribution of the ions. Now since this is so, and since my theory supposes that the make stimulation is due to the migration of the ions in the electrical field, while the break stimulation is due to the diffusion of the ions away from the anode, it is evident at once that the theory accounts for the greater effectiveness of the make stimulation.

The fact that the stronger the current the shorter the latent period, discovered by Biedermann (1879, p. 315) is in such obvious harmony with the theory that it seems hardly necessary to mention it. The velocity of the ions increases with the current, and consequently of course the distance which they must travel in order

[1] There are of course cases, such as the closing tetanus seen in winter frogs and in muscles subjected to Na_2SO_4, Na_3 citrate, etc., in which the current continues to stimulate for a much longer time after making. But as it is not certain whether this tetanus is to be attributed to the make and break twitch "substance" or to the lasting contraction "substance" it is hardly worth while to discuss the relation of this tetanus to stimulation during the flow of the current.

to stimulate is passed over more rapidly, and the latent period is diminished.

This discussion of known results might be continued almost indefinitely, but it is hardly profitable to carry it any farther as enough has already been done to show the usefulness of this theory in explaining the results of previous investigators, and in suggesting problems for future research.

Summary.

1. There are two types of response to the galvanic current which may be characterized as follows:

(*a*) Stimulation occurs only at the make and break of the current. The poles at which these stimuli occur cannot be reversed.

(*b*) Stimulation occurs also during the flow of the current; and the pole at which this stimulation takes place may be reversed.

2. The twitch of the sartorius at make and break of the constant current follows type (*a*).

3. The rhythmic fibrillary contractions of the sartorius and paramecium follow type (*b*). The behaviour of the lasting contraction (*Dauercontraction*) has not yet been definitely determined.

4. When placed in solutions the make and break twitches become modified as follows:

(*a*) In solutions of Na salts the make twitch gradually disappears, leaving only the break twitch.

(*b*) In solutions containing Na + Ca in about the proportions occurring in the blood, both make and break twitches persist as in fresh normal muscles.

(*c*) In Na + Ca solutions containing considerably more Ca than is present in the blood, the break twitches gradually disappear, leaving only the make twitches.

These facts, together with all the fundamental facts of muscle physiology, and many of its details may be explained on the basis of Loeb's theory of electrotonus by supposing that in this case stimulation must be associated with a decrease in the relative concentration of the Ca ions within the muscle.

5. The rhythmic fibrillary twitchings which occur when a sartorius muscle is placed in solutions of Na salts tend to become confined to the anode during the flow of the current.

This fact can be explained on the basis of Loeb's theory of electro-

tonus by supposing that in this case as in paramecium stimulation must be associated with a certain definite value for the relative concentration of the Ca ions in the muscle.

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THE ACTION OF PRESSOR AMINES PRODUCED BY PUTREFACTION. By H. H. DALE and W. E. DIXON.

(*From the Pharmacological Laboratory, Cambridge, and the Wellcome Physiological Research Laboratories, Herne Hill*[1].)

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I. Introductory.

The physiological activity of the amines which are formed when carbon dioxide is split off from amino-acids, by putrefaction or otherwise, has recently been recognised and has acquired interest in several distinct connexions. Abelous[2] and his co-workers first observed the rise of blood-pressure caused by intravenous injection into animals of extracts from putrid meat. The substances causing this effect have been identified by Barger and Walpole[3] as isoamylamine, phenyl-ethyl-

[1] The experiments at Cambridge were performed by W. E. Dixon: those at Herne Hill by H. H. Dale.

[2] *C. R. Soc. de Biol.* I. pp. 463, 530. 1906.

[3] This *Journal*, XXXVIII. p. 343. 1909.

amine, and *p.* hydroxyphenylethylamine. They demonstrated the origin of the last from tyrosine, and there can be little doubt that the first and second are similarly formed from leucine and phenyl-alanine respectively. In the light of their results Rosenheim[1] examined substances which he had previously prepared from certain placental extracts, which one of us (W. E. D.) with F. E. Taylor[2] had found to produce rise of blood-pressure and contraction of the pregnant uterus. He was thus able to show that one of his substances was certainly and the others probably identical with those isolated by Barger and Walpole, and that their presence was probably the result, in this case also, of incipient putrefaction. More recently G. Barger, working with one of us (H. H. D.)[3], has found that *p.* hydroxyphenylethylamine and isoamylamine are present in watery extracts of ergot, the former being responsible for almost the whole of the pressor action of the liquid extract. In the present paper we describe in detail the action of isoamylamine and *p.* hydroxyphenylethylamine, these being the most abundant of the active amines obtained from the above mentioned natural sources, and each being, moreover, a representative member of a series of related and similarly active compounds. As the actions of the two bases are in most respects similar, it will be convenient to consider them together in the case of each of the organs and tissues examined.

II. Action on the circulatory system.

As stated above the action on the blood-pressure was the first indication observed of the specific activity of these bases. We have observed the action on the circulation in cats, dogs and rabbits; the animals were in all cases anæsthetised with ether, A.C.E. mixture or urethane, or had the brain completely destroyed by pithing. No significant difference in action exists between different animals, and the action in the cat, which has been most frequently the subject of experiment, may be taken as typical for all. An experiment on man is mentioned in a later section.

Of the two amines *p.* hydroxyphenylethylamine is considerably the more active. 1 mgm. of this base, or of its hydrochloride, dissolved in water and injected intravenously causes a sudden and pronounced rise of arterial pressure reminiscent of that produced by adrenaline. The

[1] This *Journal*, xxxviii. p. 337. 1909.
[2] *Lancet*, ii. p. 1158. 1907.
[3] This *Journal*. *Proc. Phys. Soc.* May 15th, 1909.

Fig. 1. Reduce to $\frac{2}{3}$. Blood-pressure of a pithed cat. Effect of 1 c.c. $\frac{N}{10}$ isoamylamine hydrochloride, injected into the femoral vein.

Fig. 2. Same experiment as Fig. 1. Red. $\frac{2}{3}$. Effect of 2 mgms. of *p.* hydroxyphenylethylamine.

effect is in all respects slower than that of the latter substance; the blood-pressure curve shows a longer latent period, rising less rapidly to its maximum, and declining again more slowly to the original level. *p.* hydroxyphenylethylamine is many times less active than the same weight of adrenaline. An exact numerical indication showing the comparative activities is difficult, owing to the different time-relations of the actions of the two substances and the rather wide variation of individual response. In many animals, however, the activities appear to be of the order of 1 : 20. 0·05 mgm. of adrenaline will usually raise the pressure of the decerebrate animal to a higher maximum than 1 mgm. of *p.* hydroxyphenylethylamine, but the effect of the latter will last considerably longer. It should be distinctly understood, however, that this indication of relative activity only applies under the conditions given. If the drugs be administered by the mouth or subcutaneously the adrenaline loses most of its effect whilst the other amine still retains its activity. The rise of arterial pressure produced by isoamylamine has an even longer latent-period than that produced by *p.* hydroxyphenylethylamine and the first effect is often a distinct fall in pressure. The pressor action is more slowly developed and more persistent than that of *p.* hydroxyphenylethylamine, when the doses of the drugs are such as to give rise to the same maximum. Here again, individual differences of reaction make it difficult to give a numerical indication of the relative activities. Isoamylamine is, however, much less active than *p.* hydroxyphenylethylamine, and, in many animals, 1 c.c. of a $\frac{N}{10}$ solution of the hydrochloride of the former (*i.e.* 8·7 mgms. of the base) will produce an effect approximately equal to that of 2 mgms. of the latter (Figs. 1 and 2). One other feature of the action, which complicates the comparison of the activities of the bases with one another and with that of adrenaline, may be mentioned here: the effect of both becomes gradually less with repeated injections; but whereas that of *p.* hydroxyphenylethylamine declines very slowly, the effect of isoamylamine falls off rapidly. As an example of the latter effect it may be noted that, in the cat, after intravenous injections amounting in all to 10 c.c. of the $\frac{N}{10}$ solution, further injections of this base usually cause but a small response as measured by the rise of systemic blood-pressure. In determining the cause of the rise of arterial pressure the action on the heart and on the arterioles must be considered separately.

A. *The heart.* Cardiometer records show that in the case of

both bases the rise of arterial blood-pressure must be partly due to increase in the output of the heart. Fig. 3 shows the effect in the case of *p.* hydroxyphenylethylamine. The effect on the heart's activity is also well shown by adding either base to the Ringer-Locke solution whilst perfusing through the coronary circulation of an isolated heart. It will be seen from Fig. 4 that *p.* hydroxyphenylethylamine causes an almost immediate increase in the frequency and amplitude of the

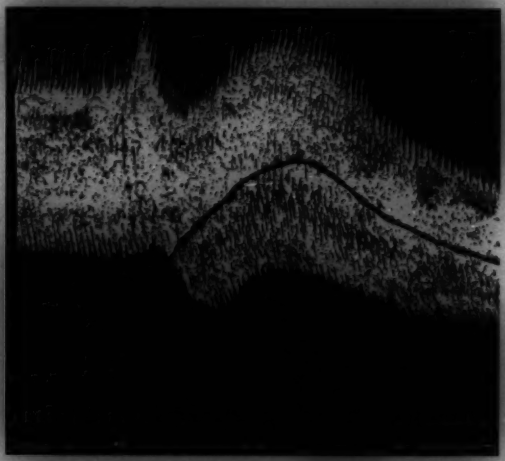


Fig. 3. Red. ⅔. Cat. Urethane. Cardiometer and blood-pressure. Effect of 1 mgm. of *p.* hydroxyphenylethylamine. Downstroke represents systole. Time=8 seconds.

ventricular beat, which persists for some time after the drug has left the circulation: it produces no initial inhibition. Isoamylamine, on the other hand (Fig. 5), produces at first a very marked decrease in the rate and vigour of the beat, which, if a sufficiently large dose has been given, may lead to temporary cessation. This initial inhibitor phase, which is represented on the blood-pressure curve by the primary fall, is succeeded by an increase in rapidity and amplitude of the heart-beat comparable to that produced by *p.* hydroxyphenylethylamine, but with the usual difference as regards dosage. The initial weakening of the beat by isoamylamine appears to be due to a direct depressant action on the heart-muscle. It is not due to action on the local vagus-

mechanism, since the effect is uninfluenced by the previous administration of atropine. It is accompanied by a marked retardation of outflow from the coronary vessels, which is too great to be accounted for by any decreased activity of the muscular walls of the heart. The coronary retardation slowly passes off and gives place to some acceleration of outflow. Both bases, when injected into the normal circulation of an animal, act like other pressor substances on the medullary centres; they cause reflex inhibition of the heart through the vagi. Section of these nerves, or their functional exclusion by atropine, is therefore necessary for the full development of the pressor effect.

Fig. 4. Red. ⅔. Isolated rabbit's heart. Effect of 0·2 mgm. of *p.* hydroxyphenylethylamine. Time = ¼ second.

Fig. 5. Red. ⅔. Same experiment as Fig. 4. Effect of 0·2 c.c. $\frac{N}{10}$ isoamylamine hydrochloride. Time = 2 seconds.

B. *The arterioles.* The participation of arterial constriction in the pressor effect of these bases can be shown by the plethysmograph, or by perfusion of isolated organs whilst recording the outflow. Fig. 6 shows a plethysmographic record from a dog's ear. Similar results were obtained with a loop of cat's intestine and with a hind limb. The vessels perfused were those of the hind limbs of a cat, of the small intestine of the dog, and of the cat's lung. The method used was

that described by Brodie and Dixon[1]. The perfusing fluid was oxygenated Ringer under constant pressure. Both bases caused marked diminution in the rate of outflow from the veins of the limbs and of the intestines. These experiments show that the vaso-constrictor effect is, at any rate to a large extent, peripheral in origin (Figs. 7 and 8). In the case of the lung the first qualitative difference in the action of the two bases was observed. *p.* hydroxyphenylethylamine resembled adrenaline in producing no constriction of the pulmonary arterioles, differing from the latter substance only in producing a less decided dilator effect on those vessels. Isoamylamine, on the other hand, produced a decided diminution

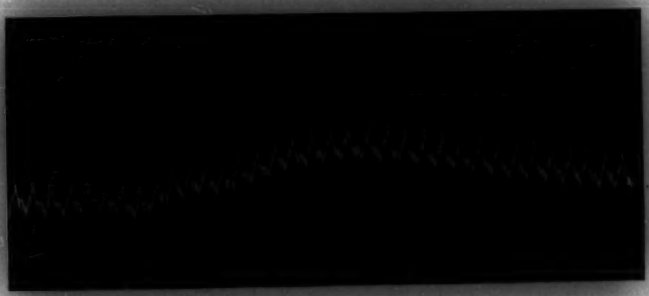


Fig. 6. Red. ¼. Dog. Morphine and urethane. Record of ear-volume and blood-pressure. Zero of blood-pressure 35 mm. below signal-line. Effect of 1 mgm. *p.* hydroxyphenylethylamine. Time = seconds.

Fig. 7. Red. ¼. Record of rate of perfusion through hind-limbs of a cat. Effect of adding 0·5 c.c. $\frac{N}{10}$ isoamylamine hydrochloride to the perfusion-fluid. Vertical lines represent the effect of adding 5 c.c. saline solution to the receiving flask. Time = seconds.

[1] This *Journal*, xxx. p. 476. 1904.

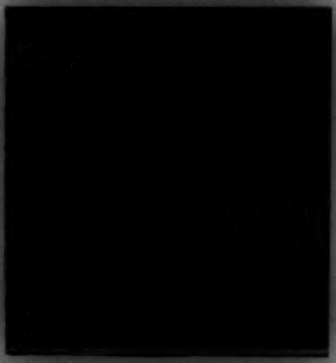


Fig. 8. Similar to Fig. 7. Red. ⅔. Perfusion through small intestine of dog. Effect of adding 5 mgms. p. hydroxyphenylethylamine to the perfusion-fluid.

Fig. 9. Similar to Figs. 7 and 8. Red. ⅔. Perfusion through lung of cat. Effect of adding 2 c.c. of $\frac{N}{10}$ isoamylamine hydrochloride to the perfusion-fluid.

of the outflow from the pulmonary veins, which could only be interpreted as indicating constriction of the arterioles. With smaller doses (1 c.c. $\frac{N}{10}$ solution) this was followed by a secondary dilation: with larger doses (2 c.c. $\frac{N}{10}$ solution) the constriction was more persistent (Fig. 9).

III. The spleen.

The muscular capsule of the spleen closely follows the arterial musculature in its reaction to drugs. It was, therefore, expected that both these bases would cause contraction of the spleen, and this was found to be the case (cf. Fig. 10). The time-relations of the effect were similar to those of the rise of blood-pressure.

Fig. 10. Red. ½. Pithed cat. Spleen-volume and blood-pressure. Effect of 2 mgms. of *p.* hydroxyphenylethylamine intravenously. Time = 10 seconds.

IV. The bronchioles.

The action on the plain muscle of the bronchioles was also examined by the plethysmographic method. It was found that *p.* hydroxyphenylethylamine had no effect whilst isoamylamine induced a very slight constriction.

V. The uterus.

In some animals, such as the rabbit, the uterine muscle resembles that of the arteries in its response to sympathetic nerve impulses and

to adrenaline[1]. Both *p.* hydroxyphenylethylamine and isoamylamine were found to cause contraction of the uterus and vagina of the rabbit in all functional conditions.

The reaction of the cat's uterus was of special interest on account of the change in response to sympathetic nerve-impulses and to adrenaline which this organ undergoes with change in its functional state, the response being inhibition of tone and rhythm in the non-pregnant condition, contraction in the pregnant condition[2]. Here again the action of both bases corresponded to that of adrenaline in that both inhibited the activity of the non-pregnant and caused contraction of the pregnant uterus of the cat. Both of them differed from adrenaline in that their motor effect on the pregnant was much more pronounced than their inhibitor effect on the non-pregnant uterus, whereas the reverse is the case with adrenaline. In a non-pregnant multiparous cat, injections of the two bases which had very pronounced effects on the blood-pressure caused but trifling inhibition of the uterine contractions. At a later stage of the same experiment, when, subsequent to large injections of nicotine, the uterus had acquired a considerable degree of tone, more marked inhibitory effects were observed with both bases: at no stage, however, in this or other

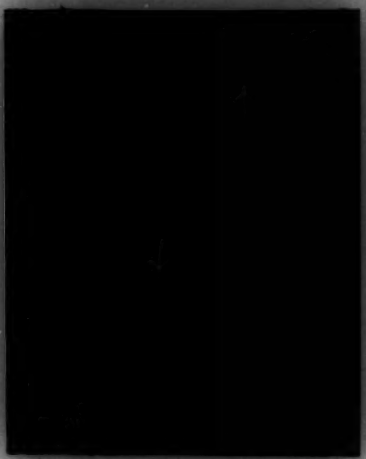


Fig. 11. Red. ½. Isolated horn of virgin cat's uterus. Effect of 1 c.c. $\frac{N}{10}$ isoamylamine hydrochloride. At ↓ the drug was added, at ↓ a change was made to pure Ringer's solution. Time = 30 seconds. Downstroke = Contraction.

[1] Langley and Anderson. This *Journal*, XIX. p. 122. 1895. Langley. This *Journal*, XXVII. p. 252. 1901.

[2] Dale. This *Journal*, XXXIV. p. 189. 1906. Cushny. This *Journal*, XXXV. p. 1. 1906. Kehrer. *Archiv f. Gynäkol.* LXXXI. p. 160. 1906.

experiments, was the inhibition induced by injection of these drugs nearly so pronounced as that caused by an injection of adrenaline, though the latter was only present in such an amount as to produce a smaller effect on the blood-pressure than those given by the bases. The virgin cat's uterus gave a more pronounced inhibitory response. Figs. 11 and 12 show records of the isolated horn of such a uterus, suspended in oxygenated Ringer's solution at 37° C. (Kehrer's method). The effect of the drug was obtained by adding 1 c.c. of $\frac{N}{10}$ isoamylamine hydrochloride and 2 mgms. of *p.* hydroxyphenylethylamine hydrochloride respectively to the 250 c.c. of Ringer's solution in the

Fig. 12. Red. ⅔. Same as Fig. 11. Effect of 2 mgms. of *p.* hydroxyphenylethylamine.

bath. By the same method was obtained the tracing, from the horn of a cat's uterus at about the middle of pregnancy, reproduced in Fig. 13. The effect of 1 c.c. of $\frac{N}{10}$ isoamylamine was practically identical. The long latent-period of the contraction, which both bases evoke in the previously quiescent organ, is probably due to the time necessary for diffusion through the greatly thickened peritoneal investment of the pregnant uterus. Change to pure Ringer's solution causes disappearance of the large rhythmic contractions which the

bases had set up and return to the original condition of moderate tonus and freedom from rhythm.

Though the pregnant uterus is thus stimulated to contraction we have not found it possible to bring on labour in pregnant animals by injection of either base. A cat near the end of pregnancy was given 5 mgm. of *p.* hydroxyphenylethylamine intramuscularly and 5 mgms. intravenously. Acceleration and strengthening of the heart-beat was observed and a strong contraction of the uterus was felt through the abdominal wall,

Fig. 13. Red. ⅔. Isolated horn of pregnant cat's uterus. Effect of 2 mgms. *p.* hydroxyphenylethylamine. At ↓ the drug was added; change to pure Ringer's solution at ↓. Time = 10 seconds. Downstroke = Contraction.

but the effects passed off and the pregnancy terminated normally a week later. A pregnant goat was given 10 mgms. of *p.* hydroxyphenylethylamine by the ear-vein: a temporary slight dyspnœa and dilatation of the pupils was the only effect observed. Later 10 c.c. of $\frac{N}{10}$ isoamylamine hydrochloride was injected intraperitoneally into the same animal, again

without inducing labour, although the pregnancy was near its normal termination.

VI. The alimentary canal.

We recorded the movements of the small intestine in the dog, under morphia and A. C. E., by the balloon method, the recorder being a Marey's tambour. The same method was used in a cat with destroyed brain. In another decerebrate cat a loop of small intestine was closed at one end, filled with warm saline and connected to a manometer filled with the same fluid: the contractions were recorded by connecting the manometer to a Marey's tambour. In all cases the result was the same, when either *p.* hydroxyphenylethylamine or isoamylamine was injected intravenously, viz. a very brief inhibition of the tone and spontaneous rhythm of the muscular walls of the intestine.

An experiment was made with an isolated loop of rabbit's intestine: again inhibition of the spontaneous rhythm was produced by adding either base to the bath of Ringer's fluid, showing that the inhibitor effect is peripheral in origin and is a genuine, primary effect, and not a secondary result of the vasoconstriction.

The musculature of the frog's stomach and intestine differs from that of the mammal in receiving its motor nerve-supply from the true sympathetic (splanchnic) nerves. It was of interest, therefore, to observe its reaction to the bases under examination. A ring of frog's stomach was suspended between hooks so that it pulled on a lever against a weak spring. It was kept moist with 0·6 % saline and, when its slow rhythmic contractions had become regular, 0·1 % *p.* hydroxyphenylethylamine in 0·6 % saline was painted on it. The effect was powerful, tonic contraction of the ring, showing that, in this case again, the effect of the base corresponds to that of sympathetic nerves and of adrenaline.

Two points of difference from adrenaline should be noted here. First the inhibition of the mammalian intestines is of a much feebler nature than that caused by adrenaline, even when the effect on the blood-pressure is approximately the same in both cases. Secondly the motor effect on the frog's stomach is relatively more pronounced than that induced by adrenaline. Comment on these facts is reserved till later.

VII. The urinary bladder.

The experiments were made on the bladder of the decerebrate cat in which the hypogastric nerves had been cut to exclude sympathetic impulses from the spinal cord or the inferior mesenteric ganglia. The volume of the bladder contents was recorded by passing a catheter, connecting this to a wide reservoir half full of water, and again connecting the top of the reservoir to a Brodie's bellows. Both bases produced distinct inhibition of the tone and rhythm of the bladder. The effect was more conspicuous in the case of *p.* hydroxyphenylethylamine, and, even in this case, was weaker than that produced by such a dose of adrenaline as caused an approximately equal rise of blood-pressure (Fig. 14).

Fig. 14. Red. ⅔. Pithed cat. Volume-record from urinary bladder and blood-pressure. At *B* 2 mgms. of *p.* hydroxyphenylethylamine were injected intravenously.

VIII. The involuntary musculature of the eye.

Intravenous injection of either *p.* hydroxyphenylethylamine or isoamylamine produces all the effects on the cat's eye and its surroundings caused by stimulation of the cervical sympathetic nerve or

injection of adrenaline, viz. dilatation of the pupil, retraction of the nictitating membrane, widening of the palpebral fissure, protrusion of the eyeball and secretion of tears. All these effects are still produced by either base and without preceptible modification, after extirpation of the superior cervical ganglion, and are, therefore, like the similar effects of adrenaline, peripheral in origin. Maximal dilatation of the pupils of enucleated frog's eyes was caused by 0·01 % *p.* hydroxyphenylethylamine and by $\frac{N}{100}$ isoamylamine.

IX. Gland cells.

It has been mentioned that both bases excite secretion of tears. In a cat into which 100 mgms. of *p.* hydroxyphenylethylamine was injected hypodermically (vid. inf.) sweating of the pads of the feet was one of the effects noted. Secretion of the submaxillary gland of the cat was excited by both bases, but the effect of isoamylamine was relatively weak in this direction, only a few drops of saliva being obtained, in one instance, after an injection sufficient to cause a considerable rise of blood-pressure. *p.* hydroxyphenylethylamine has an effect very similar to that of adrenaline, though, as in the case of the effect on the blood-pressure, it is only about 1/20 as powerful. The following records of the effects of two successive injections show the approximately equal effects produced by 5 mgms. of *p.* hydroxyphenylethylamine and 0·2 mgm. of adrenaline respectively, the activity being, therefore, in this case about 1 : 25. It will be seen that the effect of the *p.* hydroxyphenylethylamine lasts considerably the longer, though the total amounts secreted are approximately equal. The figures show, in millimetres, the movement, in successive intervals of 10 secs., of the meniscus of the saliva along a narrow glass tube, placed horizontally, and connected to a cannula in Wharton's duct. The cat was under ether.

12.41 p.m.—0·2 mgm. adrenaline by jugular vein.

0, 0, 0, 0, 1, 3, 5, 4, 5, 4, 5, 3, 3, 1, 0, 0, 0, 0, 0,.....

↑ Injection

Total :—34 mm.

12.43½ p.m.—5 mgms. *p.* hydroxyphenylethylamine by jugular vein.

0, 0, 0, 3, 5, 4, 3, 3, 2, 2, 2, 1, 1·5, 1·5, 1, 1, 1, 0, 1, 0, 0·5, 0·5, 0,

↑ Injection

1, 0, 0·5, 0·5, 0, 0, 0·5, 0·5, 0, 0, 0, 0, 0, 0,.....

Total :—36 mm.

The administration of 1 mgm. of atropine diminishes but does not eliminate this action on the salivary glands.

Preliminary extirpation of the superior cervical ganglion is without effect on the result.

The pancreatic secretion is not perceptibly influenced by either of the bases. The flow of urine from the ureters is distinctly increased by both, and this is especially marked when the blood-pressure is initially low, as in the pithed cat: the effect is probably referable to the general rise of arterial pressure.

X. Effect of certain drugs.

After successive injections of nicotine, of such magnitude (26 mgms. in all) that further injections of that alkaloid no longer influenced the blood-pressure of a decerebrate cat, each of the amines produced a rise of blood-pressure somewhat less than that which it produced originally, but still very considerable. The effect of isoamylamine appeared to

Fig. 15. Red. ⅓. Blood-pressure of pithed cat. 5 mgms. of ergotoxine previously given intravenously. Time = 10 seconds.

At *A*—0·05 mgm. adrenaline
At *B*—2 mgms. *p.* hydroxyphenylethylamine
At *C*—2 c.c. $\frac{N}{10}$ isoamylamine hydrochloride
} intravenously.

suffer a greater reduction than that of *p.* hydroxyphenylethylamine, though it is difficult to be certain on this point on account of the progressively smaller effect of repeated doses, to which we have already drawn attention. The pressor effect of both bases, however, is clearly more affected by paralytic doses of nicotine than is that of

adrenaline: that is to say their effect is less strictly, though still mainly peripheral in origin.

Ergotoxine, which annuls the motor effects of sympathetic nerves and of adrenaline, so that the pressor effect of the latter is replaced by a fall of pressure due to vasodilatation, similarly affects the action of isoamylamine and of *p.* hydroxyphenylethylamine (Fig. 15). In the case of these bases, however, the fall of blood-pressure produced after ergotoxine is insignificant. This corresponds to what we have already described, being another instance, probably, of the comparatively weak excitation of inhibitory as compared with motor sympathetic effects.

XI. Channels of absorption.

It has been shown frequently that adrenaline causes so intense a local anæmia that its absorption from the alimentary canal or even from the subcutaneous tissues is too slow to allow its general effects to be observed when it is administered by the mouth or hypodermically.

The two bases under consideration produce far less local effect: *p.* hydroxyphenylethylamine, the more powerful of the two, applied to the conjunctiva in 1:1000 solution, causes a pallor which is so slight as to need careful comparison for its detection. Both, moreover, are far more stable than adrenaline, and it is not surprising to find that they produce their general effects even when given hypodermically or by the alimentary canal. We found that 10 c.c. $\frac{N}{10}$ isoamylamine hydrochloride, injected into the rectum, produced a slow, steady rise of blood-pressure in a decerebrate cat in which the pressure had previously been slowly declining. 100 mgms. of *p.* hydroxyphenylethylamine, injected hypodermically into a cat, caused the appearance, after 2 minutes, of all the symptoms of stimulation of sympathetic nerves to an intense degree. The pupils dilated to their maximum and ceased to respond to light; there was a flow of tears and profuse salivation; the paws became moist with sweat, which could be seen exuding in beads from the pores of the hairless pads; the heart-beat became violent and interrupted, apparently by reflex vagus inhibition; respiration was rapid, the mouth being open; the sphincter ani internus was contracted to a degree which made the insertion of a clinical thermometer a matter of some difficulty. The cat showed some, but not severe prostration: the temperature rose to 104° F. The pupils began to react again to

light 30 minutes after the injection and the effects then gradually subsided. The urine, collected for the following 24 hours, contained neither albumin nor sugar and the cat remained completely normal while under observation.

An experiment upon one of ourselves, who took 10 mgms. of *p.* hydroxyphenylethylamine by the mouth on an empty stomach, indicated the rapid absorption of this base from the alimentary canal. The observation is in accordance with the clinical evidence as to the efficacy of ergot extracts so administered. The following are the readings of the arterial blood-pressure obtained at 5 minutes intervals, with Martin's Riva-Rocci apparatus.

Moving about	Resting		10 mgms. *p.* hydroxyphenyl-ethylamine in water						
120,	115,	116,	↓	124,	136,	149,	148,	135,	136,
				Micturition		30 minutes interval for a meal			
135,	135,	134,	" 134,	↓	122,	.....			130

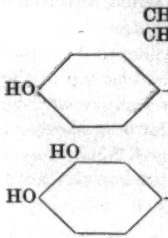
It will be seen that the pressure was still distinctly raised 85 minutes after the dose, when the readings were discontinued. During the highest portion of the rise some feeling of fulness in the head was experienced and the face was flushed: the heart-beat was powerful and slightly intermittent and the breathing became rather deeper. No salivation, perspiration or dilatation of the pupil was observed, and no nausea or malaise was produced. The urine was examined subsequently without the detection of sugar or albumin. No obvious after-effects were perceptible. The sudden necessity for micturition during the experiment suggested an increased secretion of urine, but careful controls would be needed before this could be accepted as proved.

XII. Discussion of results.

It is clear from the foregoing account that the action of both these bases has a marked similarity to that of adrenaline. Their chemical relation to that body, and to one another, as exhibited by their structural formulae (p. 43), is a clear though not a close one.

The whole question of the relationship of chemical structure to this particular action is at present under examination by one of us (H. H. D.) in conjunction with G. Barger. It is here more important to note the

differences between the actions of these bases. It has already been pointed out that the reduction of the effect of both isoamylamine and *p.* hydroxyphenylethylamine by nicotine indicates that their action is not strictly limited to the periphery. Destruction of the spinal cord similarly diminishes their effect on the blood-pressure, and, like the effect of nicotine, reduces the effect of isoamylamine more than that of *p.* hydroxyphenylethylamine. On the other hand the peripheral effect of *p.* hydroxyphenylethylamine appears to have the same limits as that of adrenaline, being restricted to such muscle-fibres and gland-cells as receive a sympathetic nerve supply, and affecting their action in the



CH_3
CH_3—$CH_1CH_2CH_2NH_2$ Isoamylamine

HO—$CH_2CH_2NH_2$ *p.* hydroxyphenylethylamine

HO
HO—$CH(OH)—CH_2NHCH_3$ Adrenaline

same sense, whether of augmentation or inhibition, as the sympathetic nerves. The peripheral effect of isoamylamine is in the main similar: in the case of structures supplied by sympathetic nerves it reproduces the action of the latter. Its action, however, is not strictly limited to such structures, since it causes constriction of the pulmonary and, probably, of the coronary arterioles. The effects of both bases, in so far as they reproduce motor actions corresponding with sympathetic excitation, are affected by ergotoxine in the same way as those of adrenaline. Both reproduce the inhibitor actions of the sympathetic system with less intensity than its motor effects.

While both bases when injected in sufficient doses into the circulation, thus produce specific effects on plain muscle and gland-cells closely resembling those of adrenaline, they differ from it in having much less general toxicity. Also their weaker local effect enables them to be absorbed much more readily from the subcutaneous tissues and through mucous membranes. The effects of sympathetic stimulation can thus be produced by these amines in a much less sudden and more persistent form. It is probable, indeed, that the effects of *p.* hydroxy-

phenylethylamine are already known and used in therapeutics, being produced by active specimens of the official ergot extracts.

The fact that the amines can be absorbed from the alimentary canal and produce their effects is also of special interest in connexion with the observation of Barger and Walpole that *p.* hydroxyphenylethylamine can be produced by allowing fæcal bacteria to grow in a medium containing tyrosine. Many observations have been published recently concerning the presence in the blood-serum and urine, in various pathological conditions, of substances which cause dilatation of the pupil of the enucleated eye of the frog. The fact that both these amines have this action casts some doubt at least on the validity of the assumption, made by certain observers, that the substance in serum, responsible for this effect, is adrenaline. It should also be mentioned, in this connexion, that both isoamylamine and *p.* hydroxyphenylethylamine have been shown to arise as the result of putrefactive changes in the cod's liver, and were, therefore, constituents of cod-liver oil prepared by the old method. *p.* hydroxyphenylethylamine has also been shown to occur in ripened cheese, being in this case again produced from tyrosine by bacterial action.

Summary.

1. Both isoamylamine and *p.* hydroxyphenylethylamine have an action which is very similar to that of adrenaline, reproducing both the motor and inhibitory effects of nerves of the true sympathetic system. Both, however, produce the motor more powerfully than the inhibitory effects.

2. Their action differs from that of adrenaline in being weaker and slower in onset, isoamylamine being much the weaker of the two, and in being less strictly though still mainly peripheral.

3. Both are absorbed from the subcutaneous tissues and the alimentary canal and produce their effects when so administered.

THE DISTRIBUTION OF PHOSPHOPROTEINS IN TISSUES. PART II.

By R. H. ADERS PLIMMER and R. KAYA.

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Plimmer and Scott[1] in their work on the distribution of phosphoproteins in tissues found that the pancreas of the ox contained a small quantity of phosphoprotein, and that the amount of phosphorus in it formed 3 per cent. of the total P_2O_5 in the organ. It was of interest to ascertain, firstly, whether phosphoprotein was present in the pancreas of the dog and what changes occurred when the pancreas was made to secrete, and secondly, whether other secreting organs such as the salivary glands contained phosphoprotein. Further, it had been found that the ova of fishes and birds contained phosphoprotein, but no experiments were made with the ova of amphibia, and in order to confirm the generalisation that phosphoproteins were present in the substances which constitute the foodstuffs of young animals an examination was made of the eggs of frogs. Phosphoprotein was not found in the testis of the ox; its absence in the testis of the codfish has now also been shown.

The method employed in carrying out these experiments was essentially the same as that adopted by Plimmer and Scott: the tissue was coagulated with alcohol; the alcohol and ether-soluble substances were extracted with hot alcohol and ether; inorganic phosphates and water-soluble phosphorus compounds were extracted with dilute hydrochloric acid and the residue after another treatment with alcohol and ether was acted upon by 1 per cent. sodium hydroxide to decompose the phosphoprotein with liberation of inorganic phosphoric acid. Determinations of the amount of the various kinds of phosphorous compounds were then made in aliquot portions of each of these extracts. As the details were the same in all the experiments it will suffice if these be given only in the first experiment and in the experiment with pancreatic juice where the quantities of P_2O_5 were so small. In all

cases the results are shown by the percentages of the various constituents. They are as follows:

A. Dog's pancreas (normal).

I. Weight of pancreas unknown.

Ethereal extract, 250 c.c. 75 c.c. contained 5·4524 mgm. P_2O_5.
That is, in pancreas 18·2 mgm. P_2O_5.

Aqueous extract, 1000 c.c. 100 c.c. contained 7·9250 mgm. P_2O_5.
That is, in pancreas 79·2 „ „

200 c.c. contained 6·9223 mgm. inorganic P_2O_5. That is, in pancreas, 34·61 mgm.

Alkali extract, 500 c.c. 50 c.c. contained 11·3486 mgm. P_2O_5.
That is, in pancreas 113·5 „ „

350 c.c. + 7 c.c. glacial acetic acid : 150 c.c. filtrate contained 1·4929 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreas, 5·08 mgm.

Total 210·9 mgm. P_2O_5.

II. Weight of pancreas (2 dogs) 40 gm.; dry residue after extraction 9·7 gm.

Ethereal extract, 100 c.c. 25 c.c. contained 22·5704 mgm. P_2O_5.
That is, in pancreas 90·3 mgm. P_2O_5.

Aqueous extract, 500 c.c. 100 c.c. contained 9·8270 mgm. P_2O_5.
That is, in pancreas 49·1 „ „

100 c.c. contained 4·4660 mgm. inorganic P_2O_5. That is, in pancreas, 22·3 mgm. P_2O_5.

Alkali extract, 500 c.c. 50 c.c. contained 17·7520 mgm. P_2O_5.
That is, in pancreas 177·5 „ „

350 c.c. + 7 c.c. glacial acetic acid : 300 c.c. filtrate contained 4·8424 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreas, 8·2 mgm.

Total 316·9 mgm. P_2O_5.

III. Weight of pancreas (3 dogs) 115 gm.; dry residue after extraction 25·3 gm.

Ethereal extract, 250 c.c. 10 c.c. contained 10·5244 mgm. P_2O_5.
That is, in pancreas 263·1 mgm. P_2O_5.

Aqueous extract, 1000 c.c. 100 c.c. contained 14·9624 mgm. P_2O_5.
That is, in pancreas 149·6 „ „

200 c.c. contained 14·3550 mgm. inorganic P_2O_5. That is, in pancreas, 71·8 mgm. P_2O_5.

Alkali extract, 750 c.c. 37·5 c.c. contained 22·3802 mgm. P_2O_5.
That is, in pancreas 447·6 „ „

650 c.c. + 13 c.c. glacial acetic acid : 200 c.c. filtrate contained 3·5728 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreas, 13·7 mgm.

Total 860·3 mgm. P_2O_5.

IV. Weight of pancreas (4 dogs) 93·4 gm.; dry residue after extraction 23·9 gm.

Ethereal extract, 250 c.c. 10 c.c. contained 8·876 mgm. P_2O_5.
That is, in pancreas 221·9 mgm. P_2O_5.

Aqueous extract, 1500 c.c. 150 c.c. contained 11·1584 mgm. P_2O_5.
That is, in pancreas 111·6 „ „

150 c.c. contained 4·9764 mgm. inorganic P_2O_5. That is, in pancreas, 49·7 mgm. P_2O_5.

Alkali extract, 600 c.c. 30 c.c. contained 22·0632 mgm. P_2O_5.
That is, in pancreas 441·3 „ „

510 c.c. + 10·2 c.c. glacial acetic acid : 200 c.c. filtrate contained 4·7212 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreas, 14·5 mgm.

Total 774·8 mgm. P_2O_5.

Hence the percentages are:

	I	II	III	IV
Ether soluble P_2O_5 ("lecithin")	8·6	28·5	30·6	28·6
Water soluble P_2O_5 (nucleic acid + inorganic)	37·6	15·5	17·4	14·4
Inorganic P_2O_5	16·4	7·1	7·8	6·4
Protein P_2O_5 (nucleoprotein + phosphoprotein)	53·8	56·0	52·0	57·0
Phosphoprotein P_2O_5	2·5	2·6	1·6	1·9

Phosphoprotein is therefore present in the pancreas of the dog and it constitutes from 2 to 2·5 per cent. of the total phosphorus in the organ. This figure corresponds closely with that found in the pancreas of the ox.

The amounts of the ether soluble and water soluble P_2O_5 in Experiment I differed considerably from those in the other three experiments; but they may be attributed to the fact that the organs in this experiment had been kept for a long time in alcohol when changes in the lecithin constituent occurred. The sum of the figures however for the ether and water soluble substances is approximately the same as in the other three experiments.

B. Dog's Pancreas (after action of secretin).

I. Weight of pancreas (2 dogs) 50 gm.; dry residue after extraction 12·5 gm.
II. Weight of pancreas (2 dogs) 60·5 gm.; dry residue after extraction 14·3 gm.
III. Weight of pancreas (4 dogs) 112 gm.; dry residue after extraction 25·9 gm.

The percentages of the various constituents were:—

	I	II	III
Ether soluble P_2O_5 ("lecithin") ...	31·1	28·0	31·0
Water soluble P_2O_5 (nucleic acid + inorganic)	16·2	19·8	16·2
Inorganic P_2O_5	7·3	6·9	6·7
Protein P_2O_5 (nucleoprotein + phosphoprotein)	52·7	52·2	52·8
Phosphoprotein P_2O_5	0·4	0·3	0

The relative amounts of the various phosphorus compounds in the pancreas after the action of secretin are thus the same as in the normal pancreas with the exception of the phosphoprotein which has diminished from about 2 per cent. to less than 0·5 per cent. of the total P_2O_5 and in the last experiment it had disappeared altogether. Phosphoprotein therefore diminishes when the pancreas is made to secrete and as will be seen it is present in the pancreatic juice which was obtained from the same dogs as used in these experiments. Except that a larger volume of alcohol was used, the method was the same as usual.

C. Pancreatic juice.

I. 88 c.c. (2 dogs B I); dry residue weighed 1·97 gm.
Ether extract, 50 c.c. 50 c.c. contained no P_2O_5.
That is, in pancreatic juice 0 mgm. P_2O_5.
Aqueous extract, 350 c.c. 140 c.c. contained 0·7608 mgm. P_2O_5.
That is, in pancreatic juice 1·9 „ „
210 c.c. contained 0·638 mgm. inorganic P_2O_5. That is, in pancreatic juice, 1·1 mgm. P_2O_5?
Alkali extract, 250 c.c. 75 c.c. contained 0·4438 mgm. P_2O_5.
That is, in pancreatic juice 1·5 „ „
175 c.c. + 6 c.c. glacial acetic acid: 150 c.c. filtrate contained 0·8868 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreatic juice, 1·5 mgm. P_2O_5.
Total 3·4 „ „

II. 178 c.c. (2 dogs B II).
Ether extract, 50 c.c. 50 c.c. contained no P_2O_5.
That is, in pancreatic juice 0 mgm. P_2O_5.
Aqueous extract, 500 c.c. 200 c.c. contained 3·804 mgm. P_2O_5.
That is, in pancreatic juice 9·5 „ „
300 c.c. contained only a trace of inorganic P_2O_5.
Alkali extract, 500 c.c. 200 c.c. contained 0·6023 mgm. P_2O_5.
That is, in pancreatic juice 1·5 „ „
300 c.c. + 12 c.c. glacial acetic acid: 200 c.c. filtrate contained 0·56782 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreatic juice, 1·5 mgm.
Total 11·0 mgm. P_2O_5.

III. 167 c.c. (4 dogs B III); dry residue after extraction weighed 3·57 gm.
Ether extract, 100 c.c. 100 c.c. contained no P_2O_5.
That is, in pancreatic juice 0 mgm. P_2O_5.
Aqueous extract, 500 c.c. 225 c.c. contained 2·0922 mgm. P_2O_5.
That is, in pancreatic juice 4·6 „ „
250 c.c. contained only a trace of inorganic P_2O_5.
Alkali extract, 300 c.c. 120 c.c. contained 1·1412 mgm. P_2O_5.
That is, in pancreatic juice 2·8 „ „
180 c.c. + 3·6 c.c. glacial acetic acid: 150 c.c. filtrate contained 1·5312 mgm. inorganic P_2O_5 (phosphoprotein). That is, in pancreatic juice, 3·1 mgm. P_2O_5.
Total 7·4 mgm. P_2O_5.

The percentages are:

	I	II	III
Ether soluble P_2O_5 ("lecithin")	0	0	0
Water soluble P_2O_5 (nucleic acid + inorganic)	56·3	86·3	59·8
Inorganic P_2O_5	present	trace	trace
Protein P_2O_5 (nucleoprotein + phosphoprotein)	43·7	13·7	36·7
Phosphoprotein P_2O_5	43·7	13·7	40·2

As the total amount of P_2O_5 in the pancreatic juice is so small, the figures representing the amounts of the various constituents must not be considered too rigidly. They show, however, that phosphoprotein is present in the pancreatic juice and that it constitutes the whole of the protein P_2O_5. Further proof of the presence of inorganic P_2O_5 in the alkali extract was given by the application of Scott's reagent[2] which showed a positive result.

If the quantities of phosphoprotein P_2O_5 in the pancreas and pancreatic juice be added together the sum amounts to only 0·5 per cent. of the total P_2O_5. When therefore the pancreas is made to secrete the principal change which occurs is destruction of phosphoprotein; its presence in pancreatic juice may be attributed rather to escape from destruction than to its forming a distinct constituent of the juice. This might be expected from the instability of phosphoprotein to the action of alkalies and tryptic enzymes.

The absence of "lecithin" in the pancreatic juice is very striking. As the ether extract contained organic matter, presumably fat or fatty acid, it is most probable that the lecithin originally present was acted upon by the lipase in the pancreatic juice and converted into water soluble P_2O_5.

D. Salivary glands (Sheep).

Weight 125 gm.: dry residue after extraction 25·4 gm.

Percentages:

Ether soluble P_2O_5 ("lecithin")	19·4
Water soluble P_2O_5 (nucleic acid + inorganic) ...	24·5
Inorganic P_2O_5	13·6
Protein P_2O_5 (nucleoprotein + phosphoprotein) ...	56·1
Phosphoprotein P_2O_5	1·8

The distribution of the phosphorus compounds in the salivary glands is very similar to that in the pancreas; in both organs protein P_2O_5 makes up over 50 per cent. of the total P_2O_5 and phosphoprotein P_2O_5 about 2 per cent.

E. Testis of Cod-fish.

I. Weight 146 gm.: dry residue after extraction 24·1 gm.
II. Weight 105 gm.: dry residue after extraction 16·1 gm.

Percentages:

	I	II
Ether soluble P_2O_5 ("lecithin")	17·8	8·9
Water soluble P_2O_5 (nucleic acid + inorganic) ...	22·6	31·9
Inorganic P_2O_5	20·5	20·4
Protein P_2O_5 (nucleoprotein + phosphoprotein) ...	59·6	59·2
Phosphoprotein P_2O_5	0	0

As expected, no phosphoprotein was present in the testis of the cod-fish, and the amount of nucleoprotein P_2O_5 was high. In Experiment II the amount of water soluble P_2O_5 is greater than in Experiment I and the amount of ether soluble P_2O_5 less: these differences are probably due to enzymatic changes in the organ taking place during the time which elapsed between the removal of the samples: sample I was taken soon after the material was obtained, sample II some hours later.

F. (*a*) *Frog's eggs* (*ovarian*).

Weight of dry residue after extraction 93·8 gm.

(*b*) *Frog's eggs* (*shortly after being laid*).

I. Weight of dry residue after extraction 5·45 gm.
II. Weight of dry substance after extraction 4·32 gm.

The percentages were:

	(*a*)	(*b*) I	(*b*) II
Ether soluble P_2O_5 ("lecithin")	26·2	19·1	21·4
Water soluble P_2O_5 (nucleic acid + inorganic P_2O_5)	4·3	13·0	9·6
Inorganic P_2O_5	0	trace	trace
Protein P_2O_5 (nucleoprotein + phosphoprotein) ...	69·5	67·9	69·0
Phosphoprotein P_2O_5	61·9	41·1	40·9

Frog's eggs therefore contain more than half their P_2O_5 in the form of phosphoprotein. The amount diminishes after they are laid, so also does the amount of "lecithin" P_2O_5, owing to the development of the embryo. In the development of the frog, just as in the chicken [3], both phosphoprotein and lecithin are made use of with an increase of the water soluble P_2O_5: only traces of inorganic P_2O_5 could be found in the early stages of the development.

SUMMARY.

Phosphoprotein is present in the normal pancreas of the dog; when the pancreas is made to secrete its amount in the gland diminishes, and it passes into the pancreatic juice where it can be detected.

Phosphoprotein is present in the salivary glands of sheep.

Phosphoprotein is absent in the testis of the cod.

Phosphoprotein is present in large quantities in frog's eggs: during their development it diminishes in amount, just as in chickens' eggs.

In conclusion, we should like to express our thanks to Prof. Starling for his kindness in helping us to procure the pancreatic juice which we required in these experiments.

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METABOLISM IN CYSTINURIA.

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Preliminary Note on Cases.

A LARGER knowledge of cystinuria has revealed the far-reaching importance of this subject. To Wollaston[1], as his name for the substance, cystic oxide, indicates, cystin was no more than a local product dependent on unknown local conditions. Some years later cystin was shown to contain sulphur[2]. In 1879 Baumann and Preusse[3] caused an experimental cystinuria in dogs by feeding them with brom-phenol and this suggested that cystin was an intermediate product of normal metabolism, and, as it contained sulphur, necessarily of protein metabolism. The identification of cystin as di α amino α thio-propionic acid by Goldmann and Baumann[4], subsequently altered to di α amino β thio-propionic acid by Friedmann[5], and Mörner's[6] preparation of cystin from hair, decided its position among the cleavage products of protein.

In 1888 diamines were discovered in the urine and fæces of a cystinuric by Udranszky and Baumann[7], and for a time the cause

of the disorder was referred to bacterial activity in the alimentary tract. This theory was soon found to be untenable. Since Baumann's time diamines have been shown to occur frequently but not always in the excreta of some cystinurics, and in many instances tyrosin and leucin have been clearly identified. It is thus usual to regard cystinuria as a disorder of protein metabolism of unknown origin, and as cystinurics are not alike in the degree to which the protein fractions are involved, it has been pointed out that the differences which exist may be referred to the absence of particular enzymes of particular tissues, employed in the catabolism of protein. In the Croonian Lectures for 1908 Dr Garrod[9] has presented a complete summary of the work on Cystinuria, and has described very fully its relationship to normal metabolism.

In the present paper there are recorded observations on three cases of cystinuria, in which special account is taken of the daily output of cystin &c. in the urine, and the presence or absence in the excreta of protein fractions other than cystin.

Case No. 1.

C. W., female, age 22 years. This subject was under close observation for a period of five weeks (March—April 1907). The case in its clinical aspect had been recorded elsewhere by others[9]. I take this opportunity for expressing my thanks to Mr Southam of Manchester for his kind help both with this case and also with W. S., the third case. The patient had been under observation for severe lumbar pain, though there was no direct evidence of renal calculus. The urine was generally slightly alkaline, normal in colour, did not contain albumin, blood or pus, and deposited the hexagonal crystals of cystin only on standing. On three occasions the urine was acid, and showed a rich deposit of cystin.

Search for Diamines. The benzoylation process of Baumann and Udranszky[7] was employed in the search for diamines, cadaverin and putrescin. On a few occasions the phenyl-isocyanate method of Loewy and Neuberg[10] was also utilised. The urine was examined daily for five weeks, and on no occasion were diamines obtained. The fæces were similarly examined on eleven occasions with negative result. The body melting at 205° C. described by Garrod and Hurtley[11] was not encountered.

Search for Tyrosin and Leucin. On no occasion, save following the administration of tyrosin, did the reaction of the urine with Millon's reagent suggest the presence of that body. On two occasions a quantity of urine (1000 c.c. and 4000 c.c. respectively) was examined with negative result for tyrosin and leucin by evaporation under reduced pressure and treatment with β-naphthalene-sulpho-chloride in accordance with the directions given by Abderhalden and Schittenhelm[12].

Search for Glycocoll. 500 c.c. urine were precipitated with phosphotungstic acid in the presence of sulphuric acid. The filtrate was freed from the reagent by baryta, and the excess barium removed by carbon dioxide. The filtrate was shaken for 6 hours with 4 gm. β-naphthalene-sulpho-chloride in ethereal solution, the reaction being kept just alkaline. The liquid was then separated from the ethereal solution of the reagent, made acid with 5N. HCl, and shaken out several times with ether. The residue obtained by distilling off the ether was extracted with boiling water. A product crystallised out, which after several re-crystallisations from hot water gave a melting point of 156° C. The amount was ·09 gm. The melting point was unaltered by the addition of a little pure naphthalene-sulpho-glycocoll. From a second 500 c.c. urine there was obtained ·02 gm. of the same product. These figures for glycocoll fall within the normal limits, so that in this case there is no apparent derangement in the catabolism of this amino-acid.

The administration of Arginin Carbonate. On April 19th the cystinuric took 5 gm. of arginin carbonate in solution, nearly neutralised by hydrochloric acid. No trace of putrescin could be detected in the urine by the benzoylation method either on this or the following day.

Professor W. H. Thompson kindly supplied the arginin carbonate.

The administration of Tyrosin. On April 6th and on April 24th the cystinuric took 5 gm. tyrosin. The tyrosin was administered in cachet, one gram every two hours. The urine on the day of the administration gave a Millon's reaction slightly more marked than that seen with normal urines. On each occasion a product was obtained by benzoylation, which after repeated re-crystallisation from alcohol gave a melting point of 253° C. This product was only obtained after the administration of tyrosin in accordance with the observations of Garrod and Hurtley[61] in another case.

The administration of Cystin. On April 12th 4 gm. of cystin, prepared from horse hair, were administered by the mouth in cachet. This was almost entirely excreted as sulphate.

The daily output of Cystin &c. On certain days quantitative estimations were undertaken. Details of this work are furnished below.

Case No. 2.

T. N., male, age 63 years, first exhibited symptoms of calculus in 1893, and was recognised at that time as a cystinuric. In the following year a suprapubic cystostomy was performed, and a cystin calculus removed. After the operation there were no further vesical symptoms. Previously the patient had had several severe attacks of gout, but since the operation only two slight attacks. I am indebted to Dr T. Armstrong Bowes of Herne Bay for this clinical note, and for his kind help in obtaining and sending samples

of urine from this case. The urine was passed into stoppered bottles containing toluene, and quantitative estimations undertaken as soon as received. The urine was faintly alkaline, pale in colour, always contained a small though variable amount of albumin, but no blood or pus. There was usually a rich deposit of cystin crystals.

In the first instance samples of urine were obtained in large bulk and independent of any particular period.

Sample 1, received April 1907. Volume 4630 c.c. Total nitrogen 31·11 gm. Total cystin 1·56 gm. C/N ratio 4·9. Tyrosin or leucin could not be demonstrated in 1500 c.c. Diamines were absent.

Sample 2, received July 25th. Volume 10550 c.c. Benzoyl cadaverin was easily obtained.

Sample 3, received Dec. 18th. Volume 10440 c.c. Total nitrogen 76·0 gm. Cystin 1·73. C/N 2·28. Diamines were not found.

Sample 4, received Jan. 27th, 1908. Diamines were not found. Diamino-acids not found.

Sample 5, received March. Diamines and diamino-acids were not found.

In Oct. and Nov. 1907 observations were made to test as far as possible any alteration in the output of cystin &c. with variation in diet.

Case No. 3.

W. S., male, age 32 years. This patient's condition was first recognised in 1906, when a cystin calculus was removed from the bladder. The clinical history of the case is recorded elsewhere by Mr Southam[6]. Samples of urine were obtained from this patient with precautions similar to those employed in the preceding case. The urine was clear, was neutral to litmus, contained no albumin, and yielded a rich deposit of cystin on standing.

Sample 1, received May 11th, 1907. Volume 4760 c.c. Total nitrogen 57·31 gm. Total cystin 1·20 gm. C/N ratio 3·5. Diamines not found.

Sample 2, received July 11th. Volume 4220 c.c. Total nitrogen 53·17 gm. Total cystin 3·08 gm. C/N ratio 5·8. Diamines not found.

Sample 3, received Dec. 5th. Volume 3840 c.c. Total nitrogen 44·82 gm. Total cystin 2·15 gm. C/N ratio 4·8. Diamines, tyrosin, and leucin not found.

In Jan. and Feb. 1908 estimations on day and night urines were carried out.

The excretion of Cystin.

1. *The daily output.* There is no satisfactory method of estimating cystin in urine. The quantity usually present is very small, so that the percentage error in estimation is considerable. The older method of

precipitation by acetic acid is valueless. Any method based on sulphur determinations assumes that the neutral sulphur in a cystinuric in excess of the normal figure occurs wholly as cystin. The β-naphthalene-sulpho-chloride method as employed by Abderhalden[26] and evaporation under reduced pressure as practised by Abderhalden and Schittenhelm[23] are complicated and therefore not free from objection. The method employed in this instance—Gaskell's[24] method, precipitation in the presence of acetone—is not absolute, and in the case of dilute urines the error is large. In such instances it was found necessary to evaporate the urine under reduced pressure, before proceeding with the estimation.

In the Croonian Lectures Garrod[6] has collected some 19 cases, in which it is possible to form an estimate of the cystin excreted. It is there seen that with one or two exceptions the average output of cystin is about 0·3 to 0·5 gm. per diem. In the later cases, where less inaccurate methods have been employed, the numbers approximate very closely. In the three cases recorded the daily excretion falls within these limits.

Case	Days	Average	Highest	Lowest
1	16	0·34 gm.	0·47 gm.	0·14 gm.
2	8	0·41	0·55	0·32
3	4	0·45	0·55	0·40

2. *The effect of diet.* In tracing the influence of diet on the output of cystin, observers have been led to rather contradictory results. Alsberg and Folin[13], relying on the absolute increase of neutral sulphur above the normal average on the same diets, came to the conclusion that on a protein-free diet the daily excretion of cystin was 0·5 gm., on a protein-rich diet 1·0 gm., *i.e.* the cystin is much increased by an increase in nitrogenous food. Abderhalden and Schittenhelm[23] by precipitation of cystin after concentration and acidification of the urine, found in one instance that the cystin was unaltered as the result of increasing the nitrogen of the food. Thiele[19] arrived at the same conclusion, but in this case also observations were restricted to single days on each diet. Recently Wolf and Shaffer[27] have confirmed the observations of Alsberg and Folin[13]. Relying on the neutral sulphur determinations without any direct cystin estimations, they conclude that the cystin of the urine varies directly as the amount of food protein.

On this occasion an attempt was made to test the effect of diet in two of the patients. Unfortunately no very wide variations in diet

could be employed. The estimations carried out in the urine were:—total nitrogen (Kjeldahl), urea nitrogen (Mörner-Sjöqvist), total sulphur, sulphate, and ethereal sulphate (Folin[58]), cystin (Gaskell[64]), and, in the second case only, creatinin (Folin[59]), uric acid (Hopkins), ammonia (Baussingault-Shaffer[60]); estimations were done in duplicate throughout.

Case 1. C. W. The patient's appetite was extremely variable, especially in the first week during catamenia. From April 4th onwards, the diet consisted approximately of: fish 150 gm. or chicken 100 gm., potatoes 100 gm., bread 200 gm., butter 50 gm., one egg, rice pudding 150 gm., milk 500 c.c., water, tea, &c. 1000 c.c.

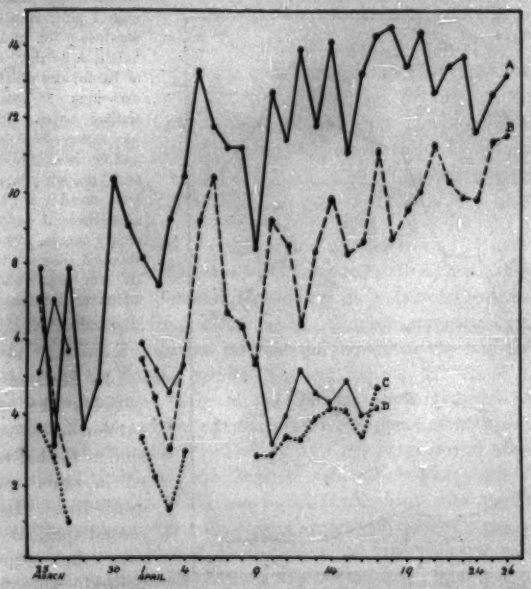

Fig. 1. C. W. The curves express the nitrogen of the food (A), the total nitrogen of the urine (B) in grams, the cystin of the urine (C) in decigrams, and the C/N ratio (D). (See Table I.)

The first diagram shows the nitrogen of the diet, the total nitrogen, cystin of the urine, the ratio $\frac{\text{Cystin} \times 100}{\text{Total Nitrogen}}$ or the C/N ratio. The

curves for food-nitrogen, and total nitrogen, of the urine undergo great variation but show partial correspondence. The cystin output, though greater on a high nitrogen diet, does not increase so much as the nitrogen output. With a sudden rise in the nitrogen as on April 10th there is a fall in the C/N ratio. The output of neutral sulphur, as given in Table I, is similar. The variation is not so wide as in the output of sulphate. Mr J. F. Gaskell very kindly undertook the cystin estimations in this case.

Case 2. T. N. In October 1907 with the co-operation of Dr Bowes I was able to obtain twenty-four hour specimens of urine on four consecutive days. During this period the patient's diet consisted of milk 1 pint, mutton ½ lb., potatoes ½ lb., bread 1 lb., butter, a herring, a rasher of bacon, and whiskey. In November I obtained a similar series. The diet was rather more generous and consisted of milk 1 pint, beef ½ lb., fish ½ lb., potatoes ½ lb., bread 1 lb., butter, 2 eggs, cheese 1 ounce, Plasmon 2 ounces. The values obtained are given in Table II. The cystin output remains very constant in spite of variations in the excretion of nitrogen.

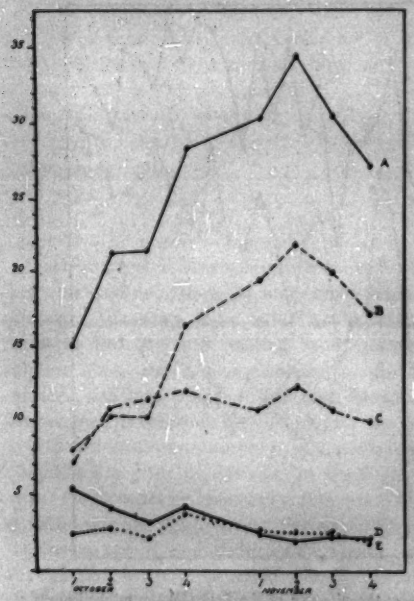

Fig. 2. Case 2. The curves express the output of total sulphur (A), total sulphate (B), neutral sulphur (C), cystin sulphur (E), in centigrams, and the C/N ratio (D). (See Table II.)

In both of these patients the neutral sulphur output is large, similar to that recorded in other cases of cystinuria. In the second patient however the output of neutral sulphur is much the higher, although the cystin is almost the same. This difference would suggest some abnormality of sulphur metabolism other than that involved in the excretion of cystin, provided there is no great error in

estimation. The influence of food on the neutral sulphur is not clear. Unfortunately no great variation could be effected. These figures therefore are not strictly comparable with those given by Alsberg and Folin[28], Wolf and Shaffer[27], who were able to employ very wide extremes of diet. Here it would appear that although the neutral sulphur tends to be higher on a high protein diet, the increase is not proportional.

Averages.	No. of days	Total nitrogen	Cystin	$\frac{\text{Cystin} \times 100}{\text{Nitrogen}}$	Neutral sulphur
Case I. C. W.	8	4·85 gm.	·26 gm.	5·5	·448 gm.
	8	8·83	·37	4·3	·578
Case II. T. N.	4	10·03	·41	4·3	1·034
	4	16·47	·38	2·3	1·098
Extremes.					
Case I. C. W.	—	2·60	·15	5·7	—
		4·06	·28	7·0	—
		11·16	·47	4·2	—
Case II. T. N.	—	6·88	·38	5·5	·702
		9·61	·44	4·3	1·090
		18·71	·39	2·1	1·250

3. *The administration of cystin.* The administration of cystin as such to cystinurics has not always been attended with a constant result. Loewy and Neuberg[31] found that while their patient was able to burn cystin obtained from calculus, he excreted unchanged cystin obtained from hair. In the first instance the increased output of sulphur was observed in the neutral sulphur fraction.

Alsberg and Folin[28] could not confirm these results. Cystin administered by the mouth was excreted as sulphate in all instances. Thiele[29] found the increase after giving cystin prepared from the patient's urine chiefly in the form of neutral sulphur other than cystin. More recently Wolf and Shaffer[27] have thoroughly investigated the problem. On all occasions cystin or cystein given by the mouth was fully oxidised, and when given subcutaneously was excreted partly as sulphate and partly as neutral sulphur.

It was only found possible to give cystin to the first patient C. W. On April 12th 4 gm. cystin (prepared from horse-hair) were administered by the mouth in cachet, one gram every two hours. Unfortunately on this day some of the urine was lost. Owing to the large variations in the diet and therefore in the excretion of sulphur-containing bodies, the effect of administering cystin is not obvious, until the percentages of

TABLE I. *Case* 1.

1907	Date	Food nitrogen	Urine vol. c.c.	Total nitrogen, gm.	Urea nitrogen, gm.	Cystin, gm.*	Urea N. ×100 / Total N.	C/N×100	Sulphur, gm. SO_3: Total	Total sulphate	Neutral	Cystin	Residual	Per cent. of total sulphur: Total sulphate	Neutral	Cystin	Residual	Per cent. of total nitrogen: Total S.	Total sulphate S.	Neutral S.	Cystin S.	Residual S.	Remarks
March	23	7·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	24	7·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	25	7·9	1014	7·12	5·88	·37	82·5	5·1	—	—	—	·24	—	—	—	—	—	—	—	—	1·4	—	
	26	3·1	520	4·06	3·60	·28	88·5	7·0	—	—	—	·19	—	—	—	—	—	—	—	—	1·9	—	
	27	7·9	525	2·60	2·41	·15	92·6	5·7	—	—	—	·10	—	—	—	—	—	—	—	—	1·5	—	
	28	3·6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	29	4·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Catamenia.
	30	10·40	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	31	9·1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
April	1	8·2	780	5·74	4·62	·34	80·4	5·9	1·198	0·614	0·584	·22	·36	51·2	48·7	18·8	29·9	8·35	4·27	4·08	1·6	2·5	
	2	7·4	580	4·30	3·70	·22	86·0	5·2	—	—	—	·15	—	—	—	—	—	—	—	—	1·4	—	
	3	9·3	475	3·03	2·66	·14	87·7	4·6	0·525	0·295	0·230	·09	·14	56·3	43·7	17·7	26·0	6·93	3·89	3·04	1·2	1·8	
	4	10·4	845	5·61	4·83	·30	86·1	5·4	—	—	—	·20	—	—	—	—	—	—	—	—	1·4	—	
	5	13·3	1360	9·17	7·68	—	85·9	—	1·754	1·213	0·541	—	—	69·2	30·8	—	—	7·65	5·29	2·37	—	—	
	6	11·6	1620	10·43	8·80	—	84·4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 gm. Tyrosin by mouth. April 6th.
	7	11·2	865	6·71	5·80	—	86·3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	8	11·2	815	6·43	5·68	—	88·3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	9	8·4	1050	5·35	4·76	·29	89·0	5·5	0·988	0·549	0·438	·19	·25	55·6	44·4	19·6	24·8	7·38	4·11	3·28	1·4	1·8	
	10	12·7	915	9·30	7·94	·29	85·4	3·2	1·847	1·248	0·595	·19	·40	67·6	32·4	10·4	22·0	7·94	5·37	2·57	0·8	1·8	
	11	11·4	870	8·57	7·45	·34	86·9	4·0	1·736	1·095	0·641	·23	·41	63·5	36·5	13·8	22·7	8·10	5·12	2·98	1·1	1·9	
	12	13·9	980	6·35	5·34	·33	84·0	5·2	2·809	2·081	0·728	·22	·51	74·1	25·9	7·7	18·2	17·69	13·11	4·58	1·4	3·2	4 gm. Cystin by mouth. April 12th.
	13	11·8	1145	8·46	7·34	·39	86·7	4·6	2·140	1·541	0·599	·26	·34	72·0	28·0	12·0	16·0	10·12	7·28	2·84	1·2	1·6	
	14	14·1	1180	9·68	8·79	·42	89·0	4·3	1·961	1·415	0·546	·28	·27	72·2	27·8	14·1	13·7	7·94	5·73	2·21	1·1	1·1	
	15	11·0	1040	8·29	7·07	·41	85·3	4·9	1·487	0·993	0·494	·27	·22	66·6	33·3	18·0	15·3	7·17	4·80	2·38	1·3	1·1	
	16	13·3	1400	8·62	7·84	·34	90·9	4·0	1·615	1·143	0·472	·23	·24	70·8	29·2	14·1	15·1	7·49	5·30	2·19	1·1	1·1	
	17	14·3	1380	11·16	9·23	·47	88·9	4·2	2·104	1·548	0·556	·31	·25	73·6	26·4	14·6	11·8	7·54	5·55	1·99	1·1	·9	
	18	14·5	800	3·69	7·82	—	89·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	19	13·4	1390	9·49	8·45	—	88·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 gm. Arginin by mouth. April 19th.
	20	14·4	1200	10·11	8·63	—	85·4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	21	12·7	830	11·29	10·18	—	89·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	22	13·4	1300	10·37	9·13	—	88·1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	23	13·7	1150	9·92	9·11	—	91·9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	24	11·6	1065	9·78	9·01	—	92·1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5 gm. Tyrosin by mouth. April 24th
	25	12·6	1250	11·26	9·55	—	86·4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	26	13·2	1075	11·50	10·20	—	88·6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	

* These Cystin estimations were carried out by Mr Gaskell.

TABLE II. *Case* 2.

Date Oct. 1907	Volume c.c.	Nitrogen, gm.						Per cent. of total nitrogen						Sulphur, gm. SO_3						Per cent. of total S.					
		Total	Urea	Ammonia	Uric acid	Creatinin	Residual	Urea	Ammonia	Uric acid	Creatinin	Residual	Cystin	C/N ×100	Total	Total sulphate	Neutral	Ethereal SO_4	Cystin	Residual	Total sulphate	Neutral	Cystin	Residual	$\frac{\text{Ethereal } SO_4}{\text{Total } SO_4} \times 100$	$\frac{\text{Total sulphur}}{\text{Total nitrogen}} \times 100$
Day 1	1300	6·88	5·60	·24	·11	·33	·59	81·46	3·53	1·60	4·76	8·63	·38	5·5	1·511	0·799	·702	—	·25	·45	52·9	47·1	16·1	30·5	—	8·78
2	1725	9·61	8·11	·42	·15	·37	·56	84·42	4·35	1·56	3·83	5·75	·44	4·3	2·136	1·046	1·090	·163	·29	·80	49·0	51·0	13·6	37·4	15·6	8·89
3	2335	10·00	8·17	·39	·14	·40	·90	81·72	3·92	1·40	4·00	8·96	·35	3·3	2·177	1·083	1·144	·176	·23	·91	47·5	52·5	10·9	41·6	17·1	8·70
4	1860	13·64	11·51	·47	·24	·49	·94	84·36	3·44	1·77	3·57	6·86	·46	4·3	2·852	1·651	1·201	·318	·39	·81	57·9	42·1	13·7	28·4	19·3	8·36
Nov.																										
Day 1	1575	15·70	13·02	·32	·21	2·15		82·94	2·06	1·34	13·70		·43	2·7	3·037	1·940	1·097	·169	·28	·81	63·90	34·1	7·2	26·9	8·72	7·74
2	1898	18·71	15·81	—	—	—		84·96	—	—	—		·39	2·1	3·449	2·199	1·250	·194	·26	·99	63·79	36·21	7·4	28·8	8·82	7·38
3	1951	16·90	14·37	·43	·20	1·90		85·00	2·55	1·18	11·27		·40	2·4	3·053	2·004	1·049	·146	·26	·78	65·65	34·35	8·7	25·7	7·27	7·22
4	1812	14·56	12·18	·36	·19	1·83		83·62	2·51	1·33	12·54		·32	2·2	2·729	1·732	·997	·149	·21	·79	63·46	36·53	7·7	28·9	8·59	7·50

sulphur in terms of nitrogen (S/N × 100) are examined (Fig. 3). There appears a large increase in the sulphur output on the cystin day, an increase almost entirely as sulphate. There is a slight rise however in the neutral sulphur of the urine, but no change in the cystin output. The excess sulphur over the average corresponds on April 12th to 2·4 gm. cystin and on April 13th to 0·8 gm. cystin, or 3·2 gm. in all. On this single occasion therefore the ingested sulphur of the cystin was almost entirely excreted as sulphate.

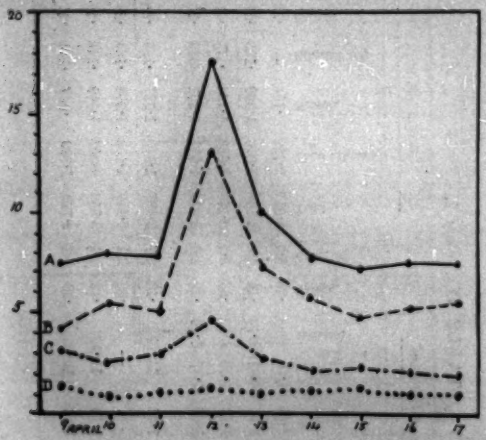

Fig. 3. Case 1. The curves represent the output of total sulphur (A), total sulphate (B), neutral sulphur (C), cystin sulphur (D), expressed as percentages of total nitrogen. On April 12th 4 gm. cystin were administered to the patient by the mouth. (See Table I.)

4. *The diurnal variation.* The recorded observations on the quantity of cystin excreted during the day and night respectively are extremely contradictory. Ebstein[28], Piccini and Conti[29] considered that the day urine contained the larger amount. Bartels[30] came to the opposite conclusion. These observers relied on precipitation tests, and their conclusions would easily be explained by differences in dilution. Wolf and Shaffer[31] give values for nitrogen, sulphur &c. in the urine over four hourly periods after the ingestion of 50 gm. casein. The outputs of neutral sulphur, total sulphur, rest nitrogen follow the output of nitrogen very closely. The maxima occur in the third period. The

maxima for ammonia and carbon are earlier in the second period. They conclude that the processes giving rise to rest nitrogen and neutral sulphur are either identical or run an absolutely parallel course. The close agreement between the neutral sulphur and total sulphur seemed

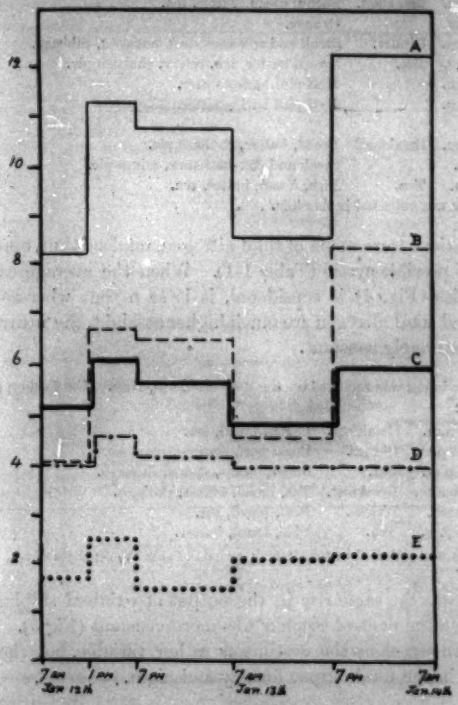

Fig. 4. Case 3. The curves show the average output per hour of total sulphur (A), total sulphate (B), neutral sulphur (D), as centigrams SO_3, of cystin (E), in centigrams, and of total nitrogen (C), in decigrams. (See Table III.)

to call for further investigation, as this agreement is not seen in the daily output with alteration of diet. I obtained therefore specimens of urine from the third case, collected over consecutive periods of the day and night. Unfortunately it was not possible to place the patient on

any standard diet. Precautions similar to those employed in the preceding instances were taken. The first experiment of this nature covered the days Jan. 12th—13th.

Food taken. Jan. 12th, 1908.		
8.30 a.m.	Breakfast.	Bacon, bread, butter, tea.
10.30 a.m.		Orange.
1.30 p.m.	Dinner.	Steak and cow-heel stew, potatoes, cabbage.
5.30 p.m.	Tea.	Bread, butter, tea, celery, rhubarb pie.
8.0 p.m.		Half pint barley water.
10.0 p.m.		Half pint barley water.
Jan. 13th.		
6.30 a.m.	Breakfast.	Bread, butter, rhubarb pie.
12 noon.		Steak and cow-heel stew, mince pie.
7.0 p.m.	Tea.	Fish, bread, butter, tea.

The urine was collected in five lots.

Estimations were made of total nitrogen, total sulphur, total sulphate and where possible cystin (Table III). When the average excretion of these bodies (Fig. 4) is considered, it is seen that whereas the total sulphur and total nitrogen are much higher at night, the neutral sulphur remains extremely constant.

This experiment was repeated on the days Feb. 9th, 10th. The food on this occasion consisted of:

Feb. 9th 9.0 a.m.	Breakfast.	Bacon, bread, tea.
1.30 p.m.	Dinner.	Roast beef, sprouts, potatoes.
6.0 p.m.	Tea.	Bread, butter, stewed rhubarb.
„ 10th 6.30 a.m.	Breakfast.	Tea, bread, butter, rhubarb.
12 noon.	Dinner.	Beef, bread, tea.
7.0 p.m.	Tea.	Fish, bread, butter.

The urine was collected in four-hourly periods (Table IV) on these days.

There was the same rise in the output of oxidised sulphur towards night, while the neutral sulphur was more constant (Fig 5).

On both occasions the cystin was rather variable, but there was no difference in the total output for day and night.

	Day	Night
Jan. 12—13th	·25	·18
„ 13—14th	·25	·26
Feb. 9—10th	—	·29 (11 hours)
„ 10—11th	·20	·20

In these experiments the increased output of total nitrogen and total sulphur towards night follows the period of increased protein intake. It is particularly interesting to notice the regular output

of neutral sulphur, an output apparently quite independent of the food taken.

Although the cystin varies from hour to hour, the difference between day and night output is very slight. Here again there is no evidence that the food has any influence on the cystin excreted.

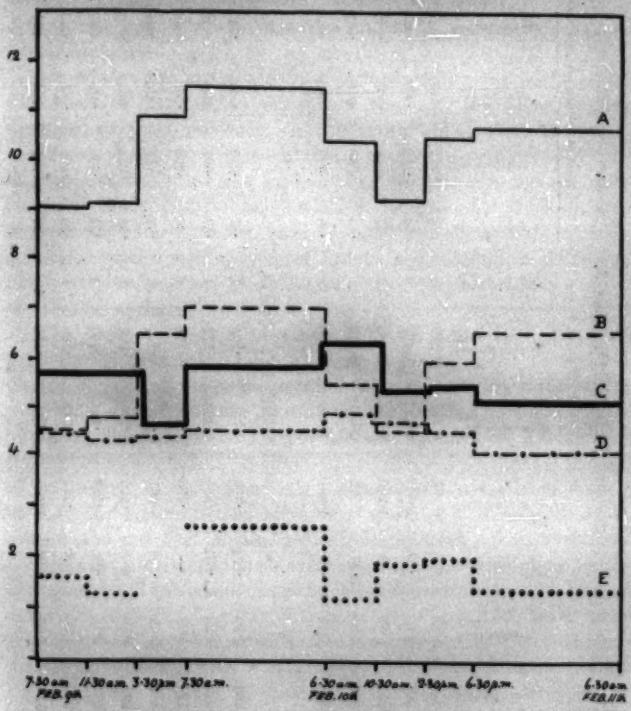

Fig. 5. Case 3. The curves represent the average output per hour of total sulphur (A), total sulphate (B), neutral sulphur (D), as centigrams SO_3, of cystin (E) in centigrams, and of total nitrogen (C) in decigrams. (See Table IV.)

The excretion of diamines.

The presence of diamines in the excreta of cystinurics has been demonstrated by a number of observers. Their occurrence in each case

TABLE III. *Case* 3.

Date		Volume c.c.	Total nitrogen, gm.	Cystin, gm.	Total sulphur as gm. SO_3	Total sulphate as gm. SO_3	Neutral sulphur as gm. SO_3	Hours of collection	Rates of excretion, gm. per hour: Total nitrogen	Total sulphur	Total sulphate	Neutral sulphur	(Cystin)	Remarks
Jan. 12th 1908	7 a.m.—1 p.m.	378	3·12	·10	0·491	0·245	0·245	6	·52	·082	·041	·041	(·017)	8.30 a.m. Breakfast.
	1 p.m.—7 p.m.	405	3·69	·15	0·677	0·403	0·274	6	·61	·113	·067	·046	(·025)	1.30 p.m. Dinner.
	7 p.m.—7 a.m.	1058	6·82	·18	1·290	0·781	0·509	12	·57	·108	·065	·042	(·015)	5.30 p.m. Tea.
Jan. 13th	7 a.m.—7 p.m.	935	5·83	·25	1·032	0·551	0·479	12	·49	·086	·046	·040	(·021)	6.30 a.m. Breakfast. 12 noon Dinner.
	7 p.m.—7 a.m.	682	7·21	·26	1·481	1·005	0·476	12	·60	·123	·084	·040	(·022)	7 p.m. Tea.

TABLE IV. *Case* 3.

Feb. 9th 1908	7.30 a.m.—11.30 a.m.	265	2·28	·064	·360	·181	·179	4	·57	·090	·045	·045	(·016)	9 a.m. Breakfast.
	11.30 a.m.— 3.30 p.m.	270	2·28	·051	·365	·193	·172	4	·57	·091	·048	·043	(·013)	1.30 p.m. Dinner.
	3.30 p.m.— 7.30 p.m.	194	1·83	—	·438	·260	·178	4	·46	·109	·065	·044	—	6 p.m. Tea.
Feb. 10th	7.30 p.m.— 6.30 a.m.	485	6·48	·286	1·261	·775	·486	11	·59	·115	·070	·045	(·026)	
	6.30 a.m.—10.30 a.m.	422	2·53	·048	·416	·220	·196	4	·63	·104	·055	·049	(·012)	6.30 a.m. Breakfast.
	10.30 a.m.— 2.30 p.m.	234	2·16	·076	·368	·181	·187	4	·54	·092	·045	·047	(·019)	12 noon Dinner.
	2.30 p.m.— 6.30 p.m.	236	2·20	·079	·422	·242	·180	4	·55	·105	·060	·045	(·020)	
Feb. 11th	6.30 p.m.— 6.30 a.m.	516	6·27	·196	1·288	·794	·494	12	·52	·107	·066	·041	(·016)	7 p.m. Tea

is very variable from day to day, but when present they are found in considerable quantity about ·3 to ·5 gm., in the 24 hours. Cadaverin is the commoner diamine in the urine, putrescin in the fæces. Cadaverin is closely related to lysin, putrescin to arginin. Loewy and Neuberg[m] found that lysin administered by the mouth to their patient was excreted as cadaverin, arginin as putrescin. Garrod and Hurtley[n] could not demonstrate any putrescin in the urine of the patient following the administration of arginin by the mouth.

1. *In the urine and in the fæces.* Although the urine was frequently examined for diamines in all three cases, and the fæces similarly examined in case 1, cadaverin was only encountered on one occasion, *i.e.* in the second sample of urine of case 2, received July 25th, 1907. On this occasion the urine was kept for three weeks under toluene before analysis. Every fraction taken yielded diamine. On benzoylation a product was obtained which, after repeated crystallisation from alcohol and water, gave a melting point of 129° C., and behaved in all respects like benzoyl-cadaverin. The yield was large, 0·5 gm. for every 1500 c.c. of urine benzoylated.

The solubility of this compound is by no means negligible. The precipitation from urine takes at least 24 hours, and if the urine is filtered immediately after benzoylation, only a small part of the benzoyl compound is present in the precipitate. On standing benzoyl-cadaverin in almost pure form is slowly deposited from the filtrate. Furthermore, the liquors from which the benzoyl-cadaverin is recrystallised is still rich in the product, so that either only a small quantity of solvent must be used, or the mother-liquors must be evaporated to small bulk on the water-bath, and the impure product so obtained again crystallised. Unfortunately benzamide is always formed together with other benzoyl compounds, and benzamide melts at the same temperature as benzoyl-cadaverin—129° C. The solubility, however, of benzamide is very much greater, so that only urines which contain a large quantity of ammonia (by decomposition of urea or otherwise) are likely to cause any trouble. No real difficulty, even in these cases, need be encountered, as the substances are very different in appearance as well as in solubility, and are readily distinguished during recrystallisation.

In this instance no benzoyl-putrescin was found but mixed with the benzoyl-cadaverin was a very small amount of impure substance melting at 196°—200° C. The quantity was too small for further purification or analysis. The product may have been identical with the compound melting at 205° C., described by Garrod and Hurtley[n] in another case.

On Dec. 8th, 1907 a third sample of urine from this patient was received. As the previous supply had been kept for some weeks before analysis, it was thought that the occurrence of cadaverin might possibly be accounted for by decomposition of diamino-acids, present in the fresh urine. Therefore, on this occasion five lots of urine, 1500 c.c. each, were taken and treated as follows:—

Lot 1. Benzoylated at once. No diamines obtained.
Lot 2. Allowed to undergo decomposition at room temperature, 6°—15° C.
Benzoylated on Jan. 24th, 1908. No diamines obtained.
Lot 3. Kept under toluene in stoppered bottle at 12°—20° C.
Benzoylated on Feb. 26th. No diamines obtained.
Lot 4. Kept under toluene in corked bottle at 12°—20° C.
Benzoylated on Feb. 26th. No diamines obtained.
Lot 5. Kept under toluene in bottle, partly closed with slit cork, at 12°—20° C.
Benzoylated on Feb. 26th. No diamines obtained.

There is no evidence that the presence of diamines in urine is connected with decomposition. Cammidge and Garrod[20] arrived at the same conclusion in 1900.

2. *The effect of diet and the administration of arginin.* Garcia[36] expressed an opinion that the quantity of diamines excreted depended on the amount of protein food. Thiele[16] concluded from single-day analyses on varied diets that the character of the food-stuffs had some effect on the excretion. The amount of benzoyl-cadaverin on a meat diet was 1·108 gm. in 24 hours, ·218 gm. in starvation, and ·366 gm. on a mixed diet. The observations are, however, too limited to carry much weight. Alsberg and Folin[13] found that on their nitrogen-rich diet their patient excreted 1·6 gm. undetermined N., as against the normal 0·6 gm. They supposed that, if this undetermined nitrogen represented amino-acids of the food that had escaped destruction, their patient on a nitrogen-free diet would excrete an amount of undetermined N., comparable with the normal figure. This they found was not so. On such a diet the undetermined nitrogen 0·8 gm. was double the normal figure of 0·4 gm. Wolf and Shaffer[17] found a similar result, 1·8 gm. on high N., ·58 on a low N. diet, the difference for the normal however in the latter instance is not so marked. There was a slight rise in undetermined nitrogen after the patient received 50 gm. casein, but no alteration in the proportion to total nitrogen. Thus there is no reason to suppose from such indirect evidence that the amount of food protein has any marked effect on the excretion of diamines. Furthermore, with the remarkable exception of the case recorded by Loewy and Neuberg[21], cystinurics have shown no intolerance to arginin or other diamino-acid.

As already stated arginin was administered to Case 1 without producing any putrescin in the urine. Nitrogen determinations were carried out on Case 2. The undetermined nitrogen was rather high, but as the diets were not properly controlled, no importance can be attached to these figures. (Table III.)

3. *Search for diamino-acids.* It was further suggested that the diamines of the urine might result from decomposition of diamino-acids excreted as such. This idea received support from the fact that in the second case the smell of cadaverin was frequently noted during evaporation of the alkaline urine before fusion, in order to make total sulphur determination, even when no diamines were detected by benzoylation. The smell became apparent after the ammonia had been driven off and before the urine began to char. Marriott and Wolf[87] in 1906 drew attention to the fact that diamino-acids had never been sought for in the urine of cystinurics. Consequently fresh urine was obtained from the patient, and examined for diamino-acids by the method employed by Wohlgemuth[88].

This may be summarised as follows :—

Four litres of urine were precipitated with basic lead acetate. The filtrate freed from lead by sulphuretted hydrogen gas, and evaporated to 1000 c.c., under reduced pressure. This was allowed to stand for seven days. No tyrosin separated.

The urine was then precipitated in the presence of sulphuric acid with phosphotungstic acid (about 1 lb. was required), and filtered—*Filtrate A*, *Precipitate A*.

Filtrate A. The phosphotungstic acid was removed by baryta, and the filtrate freed from barium by carbon dioxide followed by ammonium carbonate. The solution was then evaporated to 500 c.c. at 40° C. and 18 mm. Hg. and treated with β-naphthalene-sulphochloride for glycocoll. A small amount of impure substance was obtained, insufficient in quantity for identification.

Precipitate A. After washing, precipitate was suspended in water and decomposed by baryta. The barium phosphotungstate filtered off and the filtrate freed from barium by carbon dioxide and ammonium carbonate. The precipitation with phosphotungstic acid &c. was then repeated for further purification. After the removal of the barium, the filtrate and washings were evaporated at 40° C. and 18 mm. Hg. to 500 c.c. The liquid was treated with ammoniacal silver nitrate to remove purins, and the filtrate freed from excess of silver by sulphuretted hydrogen. After the excess of gas had been driven off, the filtrate was treated with Hopkin's mercuric sulphate reagent to remove any cystin present, and the filtrate again treated with sulphuretted hydrogen to precipitate the excess of mercury.

The liquid so obtained was then evaporated to 100 c.c. on the water-bath, and alcoholic picrolonic acid added to precipitate any arginin present. A yellow precipitate was obtained that dissolved almost at once, when washed with water, and was therefore only the precipitated reagent.

The filtrate and washings, containing excess of picrolonic acid, were evaporated to a small volume on the water-bath, shaken out several times with benzol to remove the picrolonic acid, and finally decolourised with charcoal. The colourless filtrate did not yield any permanent precipitate (lysin picrate) with picric acid.

The process was repeated on a further 6 litres of urine without producing any positive result.

Search for leucin, tyrosin, glycocoll &c.

Piccini and Conti[28] recognised tyrosin crystals in the urine of their patient, Percival[29] both tyrosin and leucin, Moreigne[30] tyrosin, although this last observer may have confused tyrosin with cystin hydrochloride. In their case Abderhalden and Schittenhelm[12] were able to identify, by full analysis, tyrosin and leucin obtained from the urine of their patient, while Fischer and Zuzuki[31] found tyrosin in a cystin calculus. These substances were sought for without success by Simon[32], Alsberg and Folin[13], Garrod and Hurtley[11]. Loewy and Neuberg[33] could only find tyrosin in the urine of their patient after the administration of tyrosin by the mouth. Simon[32], Garrod and Hurtley[11], Alsberg and Folin[13], Thiele[16] were unable to confirm this in other cases. There is no reference made in the literature to the amount of glycocoll excreted in cystinuria under ordinary circumstances. Loewy and Neuberg[33] found that their patient excreted about 20 % glycocoll unchanged after its administration by the mouth, although he could fully oxidise polypeptides containing this amino-acid.

Although tyrosin and leucin were sought for on several occasions in the three cases recorded in this paper, no trace of them could be demonstrated. The reaction of the urines with Millon's reagent did not suggest tyrosin on any occasion, save after the administration of tyrosin on both occasions to the first case C. W. On each occasion a small quantity of the "253° body" of Garrod and Hurtley[11] was obtained by benzoylation. A small quantity of glycocoll was isolated as the naphthalene-sulpho-compound from the urine of the first case, but not in excess of the normal figure.

Summary.

1. The cystin output in the three cases examined falls within the ordinary limits—·3 to ·5 gm. per diem.

2. The amount of cystin and also of neutral sulphur does alter slightly with the diet. The alteration is however slight and not in proportion to the alteration in total nitrogen and total sulphur. This is in agreement with the observations of Abderhalden[12] and of Thiele[16], but contrary to those of Alsberg and Folin[13], Shaffer

and Wolf[37]. The cases are not strictly comparable, as no wide variation in diet could be maintained in the former instances, while with Alsberg and Folin[38], and also with Shaffer and Wolf[39], the patient was placed on nitrogen-free and on nitrogen-rich diets. As a possible explanation it is suggested that the body processes, of which the excreted cystin is the last stage, require only a limited supply of food protein. This supply is early provided and only fails when the food protein is cut down to a very low figure.

3. Cystin administered by the mouth to one patient (C. W.) was excreted almost entirely as sulphate.

4. On an ordinary mixed diet the excretions of cystin and neutral sulphur are very regular through the day and night, and do not show the same marked rise towards evening as is observed in the outputs of total nitrogen and total sulphur.

5. Cadaverin was found only once in the urine of one patient (Case 2). The smell of cadaverin was frequently encountered in evaporating the urine of this patient for sulphur determination, suggesting the presence of this body in small amount. There is no evidence that decomposition of the urine has anything to do with the occurrence of diamines.

6. Arginin, given by the mouth to one patient (Case 1), was not excreted as putrescin.

7. The urine of one patient (Case 2) was searched for diamino-acids without success.

8. Glycocoll was not present in excess. Tyrosin and leucin were not found.

After giving tyrosin to one patient (Case 1) the urine gave a Millon's reaction rather more marked than the normal, and on benzoylation a small quantity of the body melting at 253° C.

This research was undertaken at the suggestion of Dr Garrod, to whom the author is much indebted for constant advice and invaluable help.

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PHYSIOLOGICAL AND HISTOLOGICAL OBSERVATIONS ON MUSCLES CHIEFLY IN RELATION TO THE ACTION OF GUANIDINE.

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The theory put forward by Langley that chemical substances do not act on the terminal branches of nerve-endings, but combine with different side-chain radicles of the contractile molecule, has recently been taken into consideration and criticised by several authors. A remarkable paper in support of a specific action on nerve-endings has been published by Führer, who was experimenting with guanidine. His results have already been discussed by Langley[1], but owing to the relation that such observations have to the general theory of the muscular contraction, the argument appeared to me worthy of an experimental criticism.

The physiological action of guanidine on the muscles has been observed by Putzeys and Swaen[2] and, almost at the same time, by Gergens and Baumann[3]. The former authors used the guanidine as the sulphate, and the latter as the carbonate; they agree that this substance does affect the muscle indirectly, *i.e.* acting on the nerve-

fibres or nerve-endings. A full account of the earlier work is given by Führer[6]. I used in my experiments guanidine chloride, of which I made every four to five days a 1·1 p.c. solution in distilled water; from that solution (which, as Führer states, is isotonic with a ·7 NaCl p.c. solution and lasts several days without alteration) the different concentrations of guanidine were obtained just before each experiment. The dilution of the stock solution was made with Ringer's fluid, and the reaction of the fluid tested with neutral litmus. No addition of alkali was found necessary to neutralise the guanidine solution, the alkalinity of the Ringer's fluid being sufficient.

The following observations have been made on *Rana temporaria*, the muscle used being chiefly the sartorius.

Experiments on normal muscles.

(*a*) *Spontaneous movements of muscles in guanidine solutions.*

In investigating the spontaneous movements of muscles in guanidine solution, I have employed the simple method of observing the behaviour of the muscle carefully dissected and immersed in solutions of different strength. The first condition that must be maintained in such experiments is that the fluid in which guanidine is dissolved must keep the muscle in good condition without acting on its physiological properties. The advantage of using Ringer's fluid needs not to be emphasised here; since however under the denomination of Ringer's fluid, solutions of different composition are employed by many physiologists, I give the composition of that which I found most suitable as an indifferent medium: NaCl 6·3, KCl ·25, $CaCl_2$ ·25, $NaHCO_3$ ·15. Distilled water 1 litre.

In general my observations agree with those of Führer. The muscle immersed in Ringer's fluid containing some guanidine presents after a certain time spontaneous movements.

The following are details of some experiments:

Exp. 1. Nov. Frog. ♂.

11.30′	R. gastrocnemius immersed in guanidine solution ·05 p.c.
11.32′ 30″	Twitching.
11.35′	Twitching more vigorously.
11.45′	Almost still.
11.50′	L. foot immersed in guanidine ·05 p.c.
11.51¼′	Twitches.
12.20′	Still twitching.
12.25′	Does not move.

12.0′ R. hand immersed in guanidine ·05 p.c.
12.5′ Movements of the fingers.
12.10′ All the muscles of the hand are twitching.
12.20′ Contractions ceased.
12.21′ The hand is put in guanidine solution ·1 p.c., twitch for two minutes.

Exp. 2. Dec. Frog. ♀.

12.17½′ L. gastrocnemius immersed in guanidine solution ·05 p.c.
12.24′ Twitches.
12.28′ Vigorous contractions of muscle.
12.36′ The twitches are less vigorous.
12.44′ Very little and seldom twitches.
12.48′ Twitching ceased.

Exp. 3. Jan.

10.10′ Right sartorius and right foot are immersed each in a dish containing ·025 p.c. guanidine solution.
10.25′ Twitching of all the foot muscles.
10.30′ Twitching of the sartorius.
The twitches of foot muscles are more frequent than those of sartorius which have a rhythm of about 3″. Movements last till 7 p.m. (8½ hours).

The action of guanidine, however, is not the same on all the muscles of the same frog, nor in all the frogs; and the difference concerns both the rapidity in the production of twitching and their strength and duration when started, while in the muscles of many frogs twitches are not seen at all. It happens also, of course, that one or more muscles of a frog twitch when put in the guanidine solution, but other muscles of the same animal do not.

Exp. 4. Dec. Frog. ♂.

(*a*) 4.14′ Left sartorius put in guanidine solution ·05 p.c.
4.15′ Very small twitches at the pelvic end of the muscle.
4.16′ The twitching stopped.
(*b*) 4.18′ Right sartorius in guanidine sol. ·1 p.c., no twitching.
(*c*) 4.24′ R. gastrocnemius in guanidine sol. ·05 p.c., no twitching.
(*d*) 4.35′ L. gastrocnemius in guanidine sol. ·05 p.c., no twitching.
(*e*) 4.44′ Rectus abdominis in guanidine sol. ·05 p.c., no twitching.

Exp. 5. Dec. Small frog.

(*a*) Both *mm. sartorii* put in guanidine sol. ·1 p.c. do not present any movements.
(*b*) 11.10′ Right foot put in guan. sol. ·1 p.c.
11.12′ Twitches vigorously.
12.15′ Twitching ceased.
(*c*) 11.15′ R. gastrocnemius put in guan. sol. ·1 p.c. The first movements appear half a minute after immersing the muscle in the fluid.
11.27′ Twitches rare but still strong.
11.35′ No more twitching.

The reasons for the different behaviour with guanidine are not very clear; it appears that fresh caught frogs are much more sensitive to its action than frogs that have been kept a long time in the laboratory. It has been already noted by Führer that young frogs are more sensitive than the old to guanidine action.

Of all the muscles of the body which have been observed in this way the foot muscles are those in which guanidine twitching is produced more quickly and more constantly; next come the hand muscles, the gastrocnemius and last of all the sartorius.

Before proceeding further it was important to understand why I found such an irregularity in production of the guanidine twitching while Führer found that twitches appear regularly in 10′—15′ (in 1 : 1000—1 : 2000 solutions). The most obvious cause lies in the composition of Ringer's fluid used by Führer. The fact that an isolated skeletal muscle in saline solution may exhibit rhythmic contractions has long been known, and in Ringer's fluid two salts are present each of which can determine that phenomenon, namely NaCl and KCl. The addition of a lime salt prevents the twitching, and the amount of Ca required for this purpose varies. Loeb[5] using a solution of NaBr found that from ·026 p.c. to ·038 p.c. of calcium chloride suffices, while Mines[6] states that ·04 p.c. or ·05 p.c. of Ca is required to stop the twitches especially in spring or summer time. With potassium chloride Mines has observed that the well-known depressant action of its salts on muscular excitability turns into an exciting action when the amount of the salt is sufficiently small, ·01—·05 p.c. The effect observed is not only an increase of excitability towards the galvanic current, but a great increase of the spontaneous twitches on addition to a NaCl solution of KCl in small proportion.

The fluid used by Führer has the following composition: $NaHCO_3$ ·1, $CaCl_2$ ·1, KCl ·075, NaCl 6·0; distilled water 1 litre. It is evident that such a fluid offers very good conditions for the production of spontaneous muscular movements, since the action of both NaCl and KCl are not prevented by the too small amount of $CaCl_2$. Using this fluid as a solvent of guanidine one cannot distinguish the twitches which are determined by guanidine from the twitches which are produced by the solvent itself. In order to ascertain the correctness of this inference I have made some experiments which gave full support to it. The record of one such experiment is given below:

Exp. 6. Dec. In three dishes I put respectively:

25 c.c. of Ringer's fluid prepared after Führer's formula.

25 c.c. of 0·5 p.c. guanidine solution in the same fluid.

25 c.c. of normal Ringer fluid. Frog ♂ pithed; right sartorius carefully dissected immersed at—

10.30′ In Führer's fluid.

10.37′ Twitches.

10.47′ The muscle is always twitching, I pass it into the normal fluid, after four to five twitches the movements are stopped.

10.57′ The muscle is immersed in Führer's fluid.

11.0′ Twitches.

11.5″ I bring the muscle into the guanidine solution in Führer's fluid, twitching continues.

It appears that spontaneous movements are produced by the simple action of the surrounding Ringer-Führer fluid without the presence of guanidine[1]. A careful observation shows that the character of the movements is different when produced by the salts of the fluid and when determined by guanidine. The latter presents often the form of a contraction *wave*, which starts from one end of the muscle (generally the pelvic) and spreads to the whole muscle length; while the salt movement presents the character of simple twitches.

The necessity of using a fluid unable to give origin to spontaneous contractions, does not need to be insisted upon. The opposite danger, that the greater quantity of CaCl may prevent and conceal the action of guanidine is avoided, using the formula given above. Führer states that 2 mol. $CaCl_2$ are required to prevent the action of 1 mol. guanidine chloride: that is about the relative amount contained in a ·01 p.c. guanidine solution in our Ringer's fluid. But his observations have been made with solutions of both substances in distilled water. The $CaCl_2$ of our fluid being scarcely sufficient to neutralise the exciting action of NaCl and KCl, it does not affect the action of guanidine, which we can therefore observe even in the lowest concentration.

The second way in which I have studied the influence of guanidine on spontaneous movements, is that of injecting into a pithed frog a solution of the substance. This method has some advantage because one is sure that the muscles are in normal conditions and all in the same conditions, so that a more exact comparison can be made of the sensitiveness of different muscles towards the substance. Injections have been made both into the aorta and into the dorsal sac, with no marked difference in the two methods.

[1] That muscles could twitch in his Ringer's fluid as well as in guanidine solution has been stated by Führer. But he does not seem to realise the importance of that fact in the interpretation of the observed twitches.

Exp. 7. Dec. Frog. ♂.

11.30′ Injection of 5 c.c. guanidine chloride sol. ·1 p.c. in the left branch of *truncus arteriosus*.
11.37′ Vigorous twitches of all the muscles of the arms and the hands.
11.39′ Twitching of *rectus abdominis*.
11.41′ Twitches of *triceps femoris*.
11.45′ Twitches of gastrocnemius.

Exp. 8. Dec. Frog. ♀.

11.15′ Injection of 2·5 c.c. guanidine sol. ·05 p.c. in the *truncus arteriosus sin.*
11.25′ No twitching. Injection of 5 c.c. same solution.
11.35′ Twitching of the *m. submaxillaris* and *obliquus abdominis*.
11.40′ Contractions of arm muscles.
11.45′ Contractions of *peronaeus*.

Exp. 9. Dec. Frog. ♂.

11.57′ Injected in the aorta 4 c.c. of a ·1 p.c. solution of guanidine.
12.1′ Twitching of the finger muscles of the left hand.
12.1′ 30″ Twitching of the finger muscles of the right hand.
12.5′ Twitches of *m. submaxillaris, rectus abdominis, flexor-radialis carpis*.
12.20′ Very energetic movements of feet muscles and gastrocnemii.
12.55′ Twitches are stopped.

Exp. 10. April. Frog. ♀.

10.9′ Injection in the dorsal sac of 5 c.c. of a ·2 p.c. sol. of guanidine.
10.17′ Twitches of *m. submaxillaris*. Movements of different muscles in this order:—Left fingers, rectus and obliquus abdominis, triceps femoris, toes, sartorius, gastrocnemius. At 2 o'clock still twitching.

Exp. 11. April.

10.44′ Injection of 5 c.c. of a 1·1 p.c. guanidine solution.
10.45′ Twitches of *mm. oculomotores*. Twitching in the various muscles in following order:—foot muscles, rectus abdominis, hand muscles, triceps femoris, arm muscles, sartorius, gastrocnemius. At 3 o'clock still twitching.

The production of spontaneous muscular movements by the guanidine action is seen more regularly in the injected animal than in the isolated muscle. Beyond the different sensitiveness shown by different kinds of muscles, the most important results of these observations are—(1) that the property of producing spontaneous contractions is more pronounced in the more concentrated solutions. The degree of the activity appears from the time which is required for producing the first twitches and from the energy of the twitches. From all the observations, of which I give only a very small number, I conclude that a 1·1 p.c. solution of guanidine produces twitches after one minute; a ·1 p.c. solution produces twitches after four to seven minutes; a ·05 p.c. solution requires 15 to 20 minutes to elicit the first twitching.

I cannot agree therefore on this point with Führer's opinion that the most suitable concentrations are those at 1 : 1000—1 : 2000, above

and below which the activity is progressively decreasing. I did not try a greater concentration than 1·1 p.c., because the hypertony of the solution would interfere with the proper action of the guanidine. But from 1·1 p.c. to ·0033 p.c. the activity of guanidine solutions, regarding the production of spontaneous twitching, is progressively decreasing. That ·01 p.c. and also ·02 p.c. solutions have a very feeble action has been already stated by Führer.

(2) It appears also that twitches in dilute solutions have a longer duration than in strong ones. This particular is more evident in the experiments in isolated muscle than in the injected frog and Exp. 3 given above is a good example of it.

(*b*) *Influence of guanidine on excitability and contractility of muscle.*

In investigating the influence of guanidine on excitability I have employed the method of finding the liminal stimulus sufficient to produce a twitch in Ringer's fluid and in the guanidine solution. The muscle (gastrocnemius or sartorius) was dissected and put in Lucas's unpolarisable electrodes modified after Mines. The construction of this apparatus is described by Mines[10]. The electrode was filled with the examined fluid and the muscle completely immersed in it. The change of the fluid is managed without disturbing the muscle by emptying the electrodes through a glass tube leading from the lowest part and admitting the new solution by the same tube. After having removed the guanidine solution the muscle was washed by allowing the Ringer's fluid to stream freely for five minutes through the electrode. The stimulus used was a break-induction shock. The graduation of the shock was obtained in the first experiments by a potentiometer of 1 m. divided in millimetres, and subsequently by a vertical induction coil of the Cambridge Scientific Instr. Co.

Exp. 12.	Dec.	Sartorius.	(*a*)	Ringer, liminal stimulus	20
				Guanidine ·1 p.c.	20
			(*b*)	Ringer	51
				Guanidine	47
Exp. 13.	Dec.	Sartorius.		Ringer, liminal stimulus	64
				Guanidine ·1 p.c.	66
Exp. 14.	Dec.	Sartorius.		Ringer, liminal stimulus	48
				Guanidine ·1 p.c.	48

Exp. 15.	Dec.	Sartorius.	(*a*) Ringer, liminal stimulus	25
			Guanidine ·05 p.c.	24
			(*b*) Guanidine	38
			Ringer	33
Exp. 16.	Dec.[1]	Liminal stimulus in Ringer		56
				56
		Liminal stimulus in guanidine ·05 p.c.		57
				56
Exp. 17.	Jan.	Liminal stimulus in Ringer		115
		,, ,, in ·025 p.c. guanidine after 10′		117
		,, ,, in ·025 p.c. ,, ,, 15′		115
		,, ,, in ·025 p.c. ,, ,, 25′		116
		,, ,, in ·025 p.c. ,, ,,		117
			Ringer	117
Exp. 18.	Feb.	Sartorius, liminal stimulus in Ringer		105
		,, ,, ,,	·011 p.c. guanidine after 25′	100
		,, ,, ,,	·011 p.c. ,, ,, 2 hrs.	95
		,, ,, ,,	·011 p.c. Ringer after 20′	97
		,, ,, ,,	·011 p.c. Ringer after 1½ hrs.	97

The general result of the observation on this point is that guanidine affects only in a small degree the excitability of the muscle, several times I found that the liminal stimulus was not appreciably changed by the change of the surrounding fluid. The examples here given show that variations, if they occur at all, may be in the direction of an increase or of a decrease of the excitability. There is no doubt that the influence of fatigue, and of other experimental conditions, can interfere and cause a certain irregularity. But I was led to suppose from the behaviour of the muscle in guanidine with mechanical stimulation that the difference observed in different experiments depends upon the different region in which the muscle had been stimulated[2]. The electrodes which I employed—that is the Lucas electrodes—allow an exact localisation of the stimulus in the region of the muscle that is brought within the constriction of the electrodes. The experiments of Kühne and others proved that the excitability of the sartorius is greater in the regions of the nerve-endings than elsewhere. The observations of Mines[(6)] allow

[1] Up to this Exp. the graduation of the stimulus is that of the potentiometer, in which the value of the current intensity increases from 0 to 100. In further experiments the graduation is that of the usual induction coil, being the distance of the secondary coil from the primary in millimetres.

[2] In the previous observations on isolated muscles I had noted that weak mechanical stimuli applied on the middle region of the muscle (immersed in guanid. sol.) do not show an increase of irritability. A stimulus applied on the ends of the sartorius often causes a series of very vigorous rhythmical contractions.

us to suppose that, where a definite change in the current needed to excite the muscle is found, there is placed the junction between neural and non-neural region of the sartorius.

With the aid of the device described by Mines in the paper already referred to I made some experiments in which the excitability of the sartorius was tested through the whole length of the muscle, by changing the localisation of the stimulus 1 mm. at a time and finding the minimal current strength needed to produce a twitch. In this way I eliminated the error due to the different localisation of the stimulus, and was able to see if guanidine acts differently on the muscle-fibres and nerve-endings. Fig. 1 illustrates the result of such an experiment performed with the sartorius of a big male frog immersed in Ringer's fluid and in a ·05 p.c. guanidine solution. On the abscissa are plotted by mm. the different points of the muscle: 0 is the pelvic and 40 the tibial end. On the ordinate are plotted the intensity of the liminal stimulus, graduated with the potentiometer.

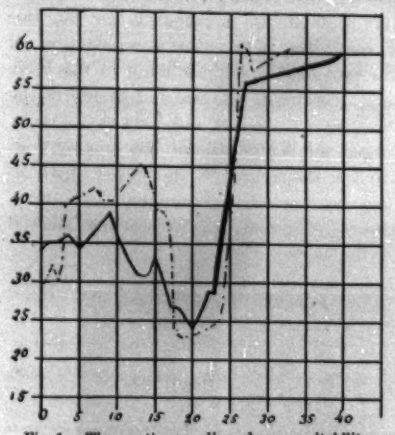

Fig. 1. The continuous line shows excitability in Ringer's fluid, the broken line excitability in guanidine.

This result explains why in the great majority of experiments where the stimulus is applied to the middle region of the muscle no remarkable change in the excitability is produced by guanidine; while sometimes a little change is seen due to some difference in the localisation of the stimulus. This experiment points out that the guanidine acts on the excitability of the proper muscle-fibre, not on the nervous devices of the muscle.

The action of guanidine on the contractility of the muscle has been investigated by comparing the response to a certain number of rhythmical maximal stimuli when immersed in Ringer fluid and when it is

immersed in a guanidine solution. The stimulus employed was a break induction shock, and the apparatus was that used in observing the excitability.

The stimulus was maximal because if it was a submaximal, an increase of the excitability producing a higher response could be interpreted like a change in the contractility. In each experiment the maximal stimulus was determined before beginning the proper observation. Each group of stimulations included 25 to 30, this number giving under good conditions of tension a regular set of contractions. The tension was provided by weights hung to the lever arm, varying from 2 to 5 grams according to the size and energy of the muscle.

A heavier load, and a larger number of stimuli was employed in some experiments to compare the fatigability of the muscle both in Ringer and guanidine solution, by tracing its ergogramm. Between every two contraction groups, or ergogramms, the muscle was allowed to rest for a time sufficient for its recovery.

Fig. 2. Red. to ½. May. Sartorius. Guanid. ·1 p.c. R = ergogramm in Ringer's fluid. G = ergogramm in guanid. sol. after 15′ immersion. Time marked in seconds. Speed of the drum ·5 mm. a second.

The influence of guanidine on the mechanical response has been investigated also, sometimes by sending to the muscle a certain number of stimulations starting from the liminal stimulus and regularly increasing the strength of the break-shock, to the maximum of the potentiometer

(5 to 10 mm. at a time). In this way the tracing presents a complex picture of the excitability and contractility changes which may occur in the muscle. The strength of the tested guanidine solutions varied from ·2 p.c. to ·001 p.c.

The record of some of such experiments is given below.

Exp. 19. Dec. Sartorius. Guanidine solution ·1 p.c.

12.0′ Muscle in Ringer's fluid. Stimulus from 64 to 98 of the potentiometer; raising one cm. at a time. Height of the contractions: minimum 26 mm., maximum 43 mm.

12.15′ Stimulus from 98 to 64 decreasing one cm. at a time. Height of the contractions, max. 44 mm., min. 26 mm.

12.16′ Change of the fluid. Guanidine solution.

12.26′ Stimulus from 66 (liminal) to 98; as above. Height of the contractions: min. 14 mm., max. 30 mm.

12.41′ Stimulus from 98 to 66. Height of the contractions: max. 30 mm., min. 10 mm.

Fig. 3. Red. to ½. Tracing of Exp. 20. R=stimuli in Ringer's fluid from 98 to 24 of the potentiometer. G=stimuli in guanid. sol. ·05 p.c. from 38 to 98 of the potentiometer. R=stimuli in Ringer from 98 to 33 of the potentiometer. Speed of the drum ·05 mm. a sec.

Exp. 20. Dec. Sartorius. Guanidine solution ·05 p.c.

11.15′ Muscle in Ringer's fluid. Stimuli from 25 (liminal) to 98 of the potentiometer, increasing one cm. at a time. Height of the contractions: min. 30 mm., max. 65 mm.

11.20′ Stimuli from 98 to 24 of the potentiometer. Height of the contractions: max. 65 mm., min. 19 mm.

12.30′ Change of the fluid: guanidine.

12.50′ Stimuli from 98 to 24 (liminal) of the potentiometer as above. Height of the contractions: max. 50 mm., min. 10 mm.

2.30′ Stimuli from 38 to 98 of the potentiometer. Height of contractions: min. 20 mm., max. 50 mm.

2.35′ Change of fluid. Ringer solution.

3.5′ Stimuli from 98 to 33. Height of the contractions: max. 65 mm., min. 25 mm.

Exp. 21. Feb. Sartorius. Guanidine chloride ·03 p.c.

11.0′ Series of stimuli in Ringer's fluid. Regular contraction. Height 20 mm.
11.10′ Change of fluid: guanidine.
11.30′ Series of stimuli. Contraction very regular. Height 20 mm.
11.45′ ,, ,, Height of contractions 20 mm.
12.40′ ,, ,, ,, ,, ,,
2.15′ ,, ,, ,, ,, ,,
2.30′ Change of fluid: Ringer's fluid.
3.30′ Series of stimuli. Height of contractions 20 mm.
4.30′ ,, ,, ,, ,, ,,

Exp. 22. Feb. Sartorius. Guanidine chloride ·02 p.c.

11.15′ Stimuli from 95 mm. (liminal stimulus) to 0 of coil distance. Height of contractions: min. 1 mm., max. 11 mm.
11.20′ Series of stimuli at 50 mm. c. d. Height of contractions, 10 mm.
11.22′ Change of fluid: guanid. sol.
11.35′ Stimuli from 95 mm. (liminal stim.) to 0 of c. d.; min. 1 mm., max. 16 mm.
11.55′ Series of stimuli at 50 mm. c. d. Height of contractions (average) 15 mm.

Exp. 23. Feb. Frog. ♂. Gastrocnemius. Guanidine ·0067 p.c. Break induction shocks: vertical induction apparatus coil distance 50 mm. Rhythm: mins. 1, 3.

2.30′ Ringer's fluid. 26 stimuli—height of contractions 31 mm. The muscle is left in Ringer's fluid 2 hours.
4.30′ 26 stimuli—height of contractions (after the first 3—4 ones, which are somewhat lower) 31 mm.
5.30′ Change of the fluid: guanidine.
6.30′ 26 stimuli—height of the contractions 39—40 mm.
6.45′ Change of fluid: Ringer's solution.
7.20′ 26 stimuli—height of contractions 28 mm.

Fig. 4. Tracing from Exp. 23.

Exp. 24. Feb. Frog. ♀. Sartorius. Guanidine ·0033 p.c.

11.40′ Muscle in Ringer's fluid. Liminal stimulus c. d. 126 mm.
11.45′ 25 stimuli at 50 mm. c.d. Height of the contractions 69 mm.
12.30′ Change of fluid: guanidine.
12.45′ Liminal stimulus, 121 mm.
12.50′ 25 stimuli at 50 mm. c.d. Height of the contractions 74 mm.

These experiments show that the action of guanidine on the contractility of the frog's muscle varies with the concentration of the substance. The action is depressing for high concentrations: from ·1 p.c. to ·03 p.c., but is an exciting one for lower concentrations: from ·03 p.c. to ·0033 p.c. A ·03 p.c. solution appears without appreciable influence on the contractility, while a ·025 p.c. shows an exciting action. The influence on the contractility is shown in the amplitude of the response to the stimulus, and those variations are parallel to the variations of the fatigability: *i.e.* in those solutions which produce a decrease in the height of the contractions the muscle is fatigued sooner than in the Ringer's fluid, and *vice versâ*.

The variations of the contractility are not associated with a condition of changed excitability. It has been already stated that guanidine does not affect, or slightly diminishes, the excitability, towards a stimulus which is applied to the neural region of the sartorius. Determining the threshold of the excitability in Ringer and in guanidine in the same muscles which were used for observations on the contractility, I observed that the same guanidine solution while remarkably affecting the work done by the muscle had no influence on its excitability. Sometimes the excitability was even somewhat lessened while the contractility was increased. Exp. 24 is an example of that.

Experiments on denervated muscle.

In order to see what share belongs to the nerve-endings in the production of the guanidine phenomena, I have taken note of the effect of denervation, on the action of guanidine, that is, I have compared the action of guanidine on normal muscles already described with its action on muscles whose peripheral innervation had been destroyed.

The following mode of procedure was used. The freshly caught frogs were washed and then anæsthetised by placing them for some minutes in very dilute ether[1]. The sciatic nerve was excised below the r. prof. ant. by removing a piece about 3—4 millimetres long, and similarly a piece of 2—3 mm. was cut away from the *ramus descendens communis*. The skin wound was sewn up, the whole operation being performed with antiseptic precautions, and the frogs, put in a tank

[1] The 2 per cent. solution of ether in water used by Professor Langley produces in the frogs effects from which sometimes they do not recover well. Employing a 1 to 1·5 p.c. solution, I had more satisfactory results in the survivals of the operated frogs.

containing fresh water, were kept in a cool dark cellar. The operations were all performed in February.

The experiments on these denervated muscles were like those described in the first part of this paper. I began them three weeks after cutting the nerves of the limb, since, according to Langley[1], Bethe and Führer[1], that is the average time required for a loss of irritability in the cut frog's nerves. During the first three weeks I made only few experiments (10, 12, 15 days after the operations) to ascertain that the degeneration of nerves was not yet begun.

(*a*) *The spontaneous movements of denervated muscles.*

Investigation of spontaneous twitching, by placing the isolated muscles in guanidine solutions, gave very uncertain results. I have already noted that guanidine twitching is a rather inconstant fact when other irritating causes are carefully avoided in the experiment. The muscles of frogs which have been kept a long time in the laboratory very seldom present twitching. Animals which are studied three weeks at least after being operated on, are from this point of view in a most unfavourable condition. Several normal sartorii of operated frogs were placed in guanidine solutions in order to observe if spontaneous movements were appearing, while the muscle of the denervated limb was used in the study of the contractility (see table below). Twitching was observed very seldom and only when the captivity life of the animal had been short.

Month	Days after the operation	Guanidine p.c.	Twitching
Feb.	7	·02	After 15'.
April	35	·05	After 10' seldom and weak.
April	36	·05	No twitching.
April	37	·1	No.
April	44	·1	No.
May	44	·05	No.
May	48	·0011	No.

The comparison between the normal and the denervated muscle of the same animal shows, that in general none of them present spontaneous twitches. Only once I could observe a twitching, and that time, both in the normal and in the denervated side. I give only a few of these negative observations.

[1] Quoted by Langley, *loc. cit.* p. 287.

Exp. 25. 53 days after nerve section. Guanidine ·05 p.c.

Normal side. Sartorius immersed in the solution 10.45′. No twitching.
Foot immersed in the solution 10.50′. 10.55′ twitches.
Denervated side. Sartorius immersed in the solution 10.40′. No twitching.
Foot immersed in the solution 10.42′. 10.49′ twitches.

Exp. 26. 51 days after nerve section. Guanidine ·05 p.c.

Normal sartorius immersed in the solution 11.25′. No twitching.
Denervated sartorius immersed in the solution 11.28′. No twitching.

Exp. 27. 43 days after nerve section. Guanidine ·05 p.c.

Normal sartorius immersed in the solution 12.18′. No twitching.
Denervated sartorius immersed in the solution 12.16′. No twitching.

The injection of guanidine in the dorsal sac or in the aorta of a frog does much more constantly produce spontaneous muscular contractions. Frogs in which the nerves of one leg have been cut, and allowed to degenerate, behave towards guanidine injection like normal animals, and no difference is noticeable between the two limbs.

Exp. 28. 59 days after nerve section.

11.29′ 5 c.c. of 1·1 p.c. guanidine injected in the aorta.
11.29′ 30″ Twitches of *M. submaxillaris.*
11.30′ *M. deltoideus* and *flexor rad. carpi.*
11.32′ Very energetic movements of flexion and extension in both legs: fibrillar twitching of all the muscles of the thighs and legs, movements of the toes.
11.55′ Strength and frequency of muscular contractions are equally diminished in both legs: still lively in the arms.

(*b*) *Guanidine action on the contractility of denervated muscles.*

The same experiments performed on the muscles of normal frogs have been repeated on the denervated muscle at various times after the operation. Sometimes comparative experiments were performed on the normal and the denervated muscle of the same animal; but as a rule only the denervated muscle was tested. The physiological experiment was associated with the histological examination of the muscular nerve-endings both in the denervated and in the normal limb. The method followed was the intra-vitam methylene-blue staining: a glass-cannula was inserted into the aorta of the frog and a $\frac{1}{60}$th p.c. methylene-blue solution in Ringer's fluid injected. The mode of procedure was that described by Eugling[7]. The muscles examined were chiefly the

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sartorii, the gastrocnemii and the foot muscles. Besides the muscles so examined in frogs, which were used exclusively for that purpose, the state of the nerve-endings was investigated in the denervated sartorii, which had been used for making graphic records.

These muscles, and the corresponding muscles of the normal side, were put on a slide, covered with a few drops of the methylene-blue solution and left in a small moist chamber, until the nerve-endings were stained.

In all cases the irritability of the cut sciatic was investigated by stimulating the isolated cut nerve with a faradic current above and below the neurome (if any).

I append some records and tracings:

Fig. 5. Red. to ½. 36 days after nerve section. Sartorius. Normal side. Guanidine ·05 p.c. Time in seconds. Coil distance 50 mm.

Exp. 29. 37 days after nerve section. Guan. ·1 p.c. Denervated sartorius.

11.15′ In Ringer 20 stimuli from 95 mm. to 0 coil distance raising 5 mm. at a time. Height of contractions, min. 7·5 mm., max. 82 mm.

11.20′ 25 break ind. shocks at 50 mm. c. d. (rhythm 1, 2 mins.). Height of contractions 55—40 mm.

11.25′ Changed fluid to guanidine solution.

11.50′ 20 stimuli from 95 mm. to 0 c.d. as above. Height of contractions, min. 5 mm., max. 51 mm.

11.55′ 25 break ind. shocks at 50 mm. c. d. Height of contractions 52—16 mm.

Fig. 6.

Fig. 8.

Fig. 6. Red. to ⅓. The same frog as in Fig. 5. Denervated side. Sartorius. Guanidine ·05 p.c. Time in seconds. Coil distance 50 mm.
Fig. 8. Sartorius denervated. 59 days. × ½. Time in secs.

Fig. 7. × ½. Sartorius. 48 days after nerve section. Guanidine ·011 p.c., load 10 grams. Influence of guanidine on the fatigue curve of muscle. Time in secs. Coil distance 50 mm.

Exp. 30. 43 days after nerve section. Guan. ·005 p.c. Denervated sartorius, load 4 grams.

12.0′	Muscle in Ringer's fluid—28 stimuli, 70 mm. c. d. Average height of contractions 50 mm.
12.10′	Change of fluid. Guanidine solution.
12.45′	28 stimuli—coil distance 70 mm., average height of contractions 55 mm.
2.30′	28 stimuli as above—average height of contractions 55 mm.
2.40′	Change of fluid. Ringer's fluid.
3.30′	28 stimulations as above—average height of contractions 47·5 mm.

Fig. 8 gives a part of the tracing obtained from the sartorius denervated 59 days, where the slight increase of the contractility shows that the muscle behaves in the usual manner even to the very dilute solution of ·0011 p.c.

Further experiments performed at various times after the denervation show that the muscle behaviour is not affected by the degeneration of the nerve-endings. Figs. 9, 10 are examples of the last experiments which I made 109 and 113 days after cutting the nerves.

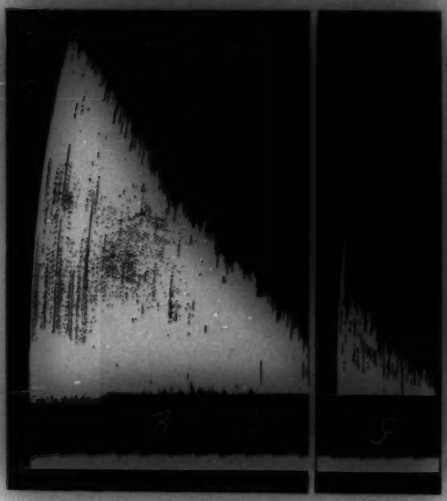

Fig. 9.

Fig. 10.

Fig. 9. Ergogramm in Ringer's fluid and in guanidine, 1 p.c. Gastrocnemius denervated 109 days. Time in secs. Rhythm 1 min. Speed of drum ·1 mm. a second.

Fig. 10. × ½. May. Gastrocnemius denervated 113 days. Guanid. ·1 p.c. Rhythm 10 secs. Time in secs. Speed of drum ·05 mm. a second.

The evidence that nerve-endings degenerate in frog's muscle has been so recently given by Langley[1] that I may dispense with an historical account and confine myself to my own observations.

Starting from the 30th day after cutting the sciatic and *ramus descendens communis* a distinct alteration is perceptible in the nerve-endings, which appear broken in several points. After 37 days not only the nerve-endings are broken but the short twigs which are still visible take on a very pale colour, and look like links of a broken chain. After 45—48 days these remnants of nerve-endings become shorter and shorter and their number in the muscle is remarkably decreased. After 50 days the methylene-blue no longer reveals any nerve-endings. The nerve plexus on the upper (pelvic) artery of sartorius does not degenerate. No trace of regeneration was to be seen in 109 and in 113 days, *i.e.* about two months after the complete disappearance of the nerve-endings.

The cut sciatic is throughout this period quite unexcitable, and this fact is in accordance with the results of histological observation.

The action of muscle extract on guanidine.

As the previous experiments suggested that the action of guanidine on the muscles is independent of the presence of their nerve-endings, I tried to obtain a direct demonstration of the relations between guanidine action and the muscular substance.

An extract of frog muscles was made by grinding down a certain quantity of them with sand and guanidine solution. In Ringer's fluid the extract was filtered and its action on the muscular contractility tested in the usual way. The muscles used for making the extract were always the sartorius and triceps femoris, the weight being about 1 gr.

The experiments show that the extract of 1 gram of muscle mixed with 100 c.c. of ·05 p.c. guanidine solution prevents the usual depressing action of that solution. Fig. 11 reproduces a portion of the tracings obtained under such conditions. The second series of contractions, obtained after 15 mins. immersion of the muscle in muscle extract and guanidine, shows that the contractility of the muscle is not appreciably changed, while the two following series present the usual very distinct decrease produced by the ·05 guanidine solution, after 15 and 31 minutes immersion.

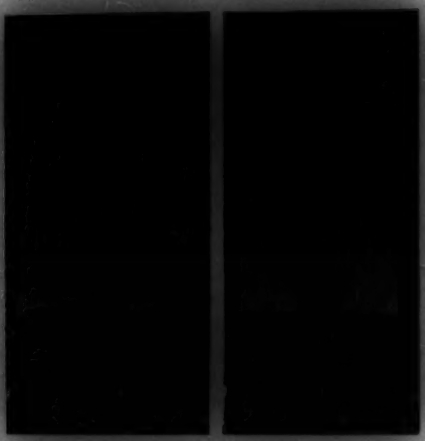

Fig. 11. Red. to ⅓. Gastrocnemius. Comparative action of guanidine ·05 p.c. in muscle extract (G. M.) and guanidine in Ringer (G.) on contractility. Time in secs. Load 5 grams. Coil distance 50 mm.

The following is another experiment of the same kind:

Exp. 31. May. Sartorius of ♂ frog.

11.0′	Muscle in Ringer's fluid.
11.5′—10′	Series of 30 stimuli at 60 c. d. Rhythm 10 secs. Average height of contractions 50 mm.
11.13′	Change of fluid. Muscle extract (1 gr. in 100 c.c. of a ·05 p.c. guanid. sol).
11.28′—33′	Series of 30 stimuli as above. Average height of contractions 50 mm.
11.45′	Series of stimuli as above. Average height of contractions 50 mm.
11.55′	Change of fluid. ·05 p.c. guanidine in Ringer.
12.15′	Series of 30 stimuli as above. Height of contractions 41 mm.
12.25′	Change of fluid. Guanidine and muscle extract.
12.35′	Series of stimuli as above. Height of contractions 44 mm.
12.55′	Change of fluid. Guanidine in Ringer.
1.10′	Series of stimuli as above. Height of contractions 38 mm.

The extract of the same weight of muscles made up with 100 c.c. of ·1 p.c. guanidine solution has an exciting action. Fig. 12 reproduces partially the graphic record of an experiment of this kind (April) in which the exciting action is very remarkable. We have seen that a ·1 p.c. guanidine solution in Ringer is depressant, and in the same figure is seen the usual effect of this solution in the groups of contractions marked

with G. Since the action of guanidine is an exciting one in low concentrations, it seems probable that a part of the substance combines with some substance of the muscle extract, so that the solution becomes very dilute and acts accordingly.

These facts have been observed in several experiments. I give another example in Fig. 13 where the contractility of a sartorius is recorded in guanidine, in Ringer's fluid and in guanidine + muscle extract again.

Fig. 12. Red. to ½. April. Gastrocnemius. Comparative action of guanidine in Ringer's fluid (G.) and guanidine in muscle extract (G. M.) on contractility. The first group, R., is the contraction in Ringer's fluid. Time in seconds. Load 5 grams. Coil distance 50 mm.

Fig. 13. Red. to ⅔. May. Sartorius. Details as in Fig. 12. Speed of the drum ·05 mm. a second.

The extract of the same weight of muscle, made up in 100 c.c. of Ringer's fluids does not act on the contractility.

The change which is observed in the action of guanidine does not therefore depend upon an influence of the muscle extract itself on the contractility. It is the guanidine that is influenced by some constituent of the muscle, probably by a chemical combination comparable with that which occurs, according to Ehrlich's theory, between receptors and toxins or such a combination as has been recently demonstrated by Dixon and Hamill[19] as occurring between secretin and some constituents of the pancreas.

Discussion of the results.

From the facts recorded a clear idea is obtained of the mode of action of guanidine.

The spontaneous movement produced by immersing an isolated frog muscle in a guanidine solution, or by injecting a certain quantity of the same solution into a frog, is a phenomenon of different order from the action on the contractility of the muscle. This fact is demonstrated by the influence of concentration in both cases, i.e. the property of eliciting spontaneous movements decreases progressively when the concentration of the solution is decreasing from 1·1 p.c. to ·003 p.c., while the action on the contractility changes its direction along the scale of concentrations, being a depressant one of from ·1 p.c. to ·03 p.c. and an exciting one from ·03 p.c. to ·0011 p.c.

These facts indicate that guanidine acts on two different substances of the muscle fibre, one of which is responsible for the spontaneous twitches and the other for the mechanical response to an electric stimulus.

The twitching property of the isolated muscle when in presence of guanidine is, in the frog muscle, of such a delicate nature that it can be lost by slight change of condition, such as the confinement of the animal in the laboratory. It seems that in these abnormal conditions of life some substance of the muscle fibre disappears. But frogs kept even for a long time in the laboratory present spontaneous twitches in their skeletal muscles if, after being pithed, they are injected with a guanidine solution.

The action of guanidine on the excitability towards direct stimuli tested on the sartorius, is seen to be different in the different regions of muscle. The excitability of the neural region is unaffected or

decreased, while in the regions of muscles where there are no nerves it is increased.

This increase of the excitability in the muscular region may be seen in several cases when the contractility of the muscle is depressed; so that it seems natural to think that production of spontaneous twitching and increase of excitability are two manifestations of the same action of guanidine, distinct from its action on the muscular contractility. The topographical difference, which we have noted, indicates that the action of guanidine, so far as it concerns twitches and excitability, has its seat in the muscle fibres and not in the nerve-endings. But on this point a more direct and clear conclusion is given by the experiments on the denervated muscle. The guanidine has on the contractility of the muscle a very regular and constant action which can be followed with exactness in all the concentrations of the substance, and which has a very characteristic feature in the change of direction which the action undergoes at a definite concentration. This action being observed, without any difference in the denervated muscles when their nerve-endings are degenerated, it is clear that guanidine acts on muscle fibres and not on nerve-endings.

The conclusion of Führer, that guanidine acts on nerve-endings is supported by a complicated interpretation of his experiences which is difficult to accept. A theoretical criticism of this view which admits a regeneration of nerve-endings without any connection with the central nervous system, and which allows that some nerve-endings may be regenerated several days before others are completely degenerated, has been made already by Langley[1].

My observations give the direct demonstration that guanidine action is the same on muscles in which all the nerve-endings are degenerated and no trace of regeneration is to be seen as it is on normal muscles. I may add that Führer's experiments regarding spontaneous twitching do not allow of any definite conclusion, since we have seen that guanidine twitching is a very inconstant phenomenon in frogs which have been kept some time in the laboratory, and that the Ringer's solution employed by Führer is able to produce spontaneous movements independently of guanidine.

The last part of my experiments show that guanidine mixed with muscle extract loses its normal action on the muscular contractility. The idea that this fact depends upon a combination of guanidine with some constituent of the muscle is supported by the quantitative relation between guanidine and muscle extract. The same quantity of muscle

extract which abolishes the guanidine action of 100 c.c. of a ·05 p.c. solution, changes the depressant action of 100 c.c. of a ·1 p.c. solution into an exciting one, *i.e.* the muscular solution, combining only with a portion of the guanidine, changes the concentrated depressing solution into a dilute, and therefore exciting solution.

The view that a combination takes place, between guanidine and some constituent of muscle, is not open to the objections which have been brought against other experiments supporting the conception of the existence of chemo-receptors for drugs. The mixture of muscle extract and guanidine being filtered, the delay in the absorption of the substance can have but little importance in the loss of the characteristic features of guanidine. As a matter of fact, the experiments showed that the contractility of a muscle, in Ringer's fluid, is the same as that of a muscle in Ringer's muscle extract $\left(\frac{1 \text{ gr.}}{100 \text{ c.c.}}\right)$, so that in this extract the diffusion and absorption of salts is the same as in the Ringer's fluid.

It has been urged by Dixon and Hamill that the experiment supporting the side-chain hypothesis yields a negative result when applied to vegetable drugs. Guanidine being a product of the animal body this criticism is not applicable to our case, and I am inclined to think that a combination of guanidine with the protoplasmic molecule of the muscle is a fact of the same order as the combination of toxins with the toxin-receptors, or of secretin with the precursors of pancreatic enzymes.

Summary.

(1) Guanidine acts on the frog muscles by producing spontaneous twitching and by affecting their contractility.

(2) The production of spontaneous twitches is an inconstant phenomenon, which can be seen also on denervated muscles.

(3) The action on contractility is the same in normal muscles and in muscle whose nerve-endings are completely degenerated.

(4) Guanidine acts by combining with two different substances of the muscle, one of which is responsible for the twitches, the other for the changes in contractility.

I wish to express my sincere thanks to Prof. Langley for the kindness of the hospitality which I found in his laboratory, and for the invaluable help which he gave me by suggesting the argument of these researches, and giving me his criticism and encouragement. My thanks

are due also to the other physiologists of the Cambridge Laboratory, especially to Mr K. Lucas and Mr G. R. Mines for the kindness with which they made my work easy by the loan of several pieces of apparatus.

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A METABOLISM EXPERIMENT WITH SPECIAL REFERENCE TO THE ORIGIN OF URIC ACID.

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COMPLETE or nearly complete analyses of the products of metabolism have only been carried out by Folin[1] and by Cathcart[2]. The former, by improved methods, has given us an insight into the metabolism of normal individuals on a constant diet, the latter into that of a starving individual. The other investigators have devoted their attention mainly to the elucidation of the metabolism of separate items in the body, chiefly that of uric acid. H. Wiener collected their results in his article on uric acid in the *Ergebnisse der Physiologie* in 1902 and we now regard the uric acid in the urine as partly exogenous and partly endogenous in its origin; both are referred back to purines, the exogenous to the purines in the food, the endogenous to the purines in the body. With regard to the exogenous moiety the total quantity of purines administered with the food never appears as uric acid but only from one-quarter to one-half of its amount. There would seem, so far as we can discover, to be only one exception to this generalised statement, namely that by Hopkins and Hope[3] who found an increase in the output of uric acid when an almost purine-free food was administered.

It was therefore thought that by making analyses of *all* the more important nitrogenous constituents in the urine and by maintaining the subject of the experiment for at least one week upon each of three selected diets, (1) a meat diet, (2) a purine-free diet, (3) a purine-rich diet in which only the protein constituent was varied and by administering this diet at the usual meal times of the subject under investigation a more definite conclusion as to the origin of uric acid in the body might be arrived at.

The subject of the experiment was a healthy man aged 39 (one of us, M. D.).

The three selected diets were chosen so that each yielded 110 gm. protein, 240 gm. carbohydrate and 100 gm. fat per diem (Voit's standard). The carbohydrate and fat constituents consisted of potato and butter; these were preferred to the usual bread diet by the subject of the experiment. The protein constituents consisted of (1) beefsteak, (2) egg-white, (3) herring roe. Hard-boiled egg-white was chosen in preference to egg and milk, the usual purine-free diet given, on account of its freedom from fat, lecithin etc., and on account of its simpler protein nature. Herring roe was chosen on account of the ease with which it can be obtained ready for food and on account of its great richness in purines. On this diet a slight variation in the amount of butter administered should have been made in accordance with its fat content, but this was not done as the extra amount of fat thus taken was so small in comparison with the total intake.

The amounts of each food administered were calculated from analyses made of the meat, egg-white and herring roe, namely

1 gm. beefsteak contained 0·0350 gm. N = 3·5 per cent.
= 21·87 per cent. protein,

1 gm. egg-white contained 0·0203 gm. N = 2·0 per cent.
= 12·68 per cent. protein,

1 gm. herring roe contained 0·03402 gm. N = 3·4 per cent.
= 21·42 per cent. protein,

and from the tables published in König's *Nahrungs- und Genussmittel.* The quantities taken were:

Diet I.

	Breakfast		Lunch		Dinner				Protein	Carbohydrate	Fat
Beefsteak	0	+	250	+	250	=	500 gm.	=	100	—	26
Potato	200	+	300	+	300	=	800 ,,	=	16	160	—
Butter	30	+	35	+	35	=	100 ,,	=	—	—	80
Sugar	20	+	20	+	20+20	=	80 ,,	=	—	80	—
									116	240	106

Diet II.

Egg-white	100	+	350	+	350	=	800 gm.	=	100	—	—
Potato	200	+	300	+	300	=	800 ,,	=	16	160	—
Butter	30	+	50	+	50	=	130 ,,	=	—	—	104
Sugar	20	+	20	+	20+20	=	80 ,,	=	—	80	—
									116	240	104

TABLE I.

Date	Diet	Volume	Sp. gr.	Acidity N/10	Total P_2O_5, gm.	Inorg. P_2O_5, gm.	Org. P_2O_5, gm.	Urea, gm.	Ammonia, gm.	Creatinine, gm.	Creatine, gm.	Uric acid, gm.	Body weight, kilogm.	Stools passed
Feb. 12	Mixed	1840	—	696	2·84	2·55	0·29	—	—	—	0	0·76	75·8	10.45 p.m.
13	Mixed	1150	—	568	2·59	2·60	0·0	—	0·50	1·32	0	0·83	75·8	
14	Mixed	2050	—	—	2·81	2·64	0·17	30·45	0·51	1·46	0	0·98	75·8	7.45 a.m.
15	Mixed	1000	1025	736	2·71	2·43	0·28	26·80	0·49	1·32	0	0·69	76·5	8.30 p.m.
16	Mixed	1170	1022	—	2·69	2·62	0·07	30·09	0·52	1·41	0	0·79	76·0	8.0 a.m.
17	Mixed	1120	1023	—	2·89	2·93	0·0	31·24	0·54	1·54	0	1·10	75·8	
18	Mixed	1250	1021	608	2·59	2·58	0·01	32·46	0·53	1·28	0	0·86	76·2	7.40 a.m.
				652	2·76	2·61	0·12	30·35	0·52	1·39		0·86		
Feb. 19	I	1460	1019	488	2·74	2·49	0·25	32·08	0·53	1·40	0	0·84	76·0	
20	I	1600	1016	352	2·26	2·32	0·0	31·76	0·45	1·38	0·23	0·88	75·3	7.45 a.m.
21	I	1075	1022	432	2·43	2·27	0·16	31·22	0·46	1·27	0·23	0·80	75·3	
22	I	1420	1018	432	2·28	2·20	0·08	33·30	0·39	1·34	0·05	0·75	75·0	
23	I	1150	1022	480	2·51	2·42	0·09	30·26	0·39	1·53	0·28	0·76	75·0	
24	I	1100	1020	472	2·23	2·10	0·13	30·24	0·40	1·39	0·22	0·76	75·0	8.10 p.m.
25	I	1445	1017	512	2·46	2·40	0·06	34·11	0·41	1·38	0·16	0·75	75·6	
				453	2·42	2·31	0·11	31·85	0·43	1·38		0·79		
26	II	1745	1015	352	1·34	1·26	0·08	31·48	0·32	1·09	0	0·56	75·0	10.15 p.m.
27	II	1800	1015	272	0·94	0·98	0·0	32·64	0·33	1·05	0	0·55	74·6	
28	II	1980	1015	272	1·09	1·17	0·0	32·27	0·35	1·23	0	0·48	74·2	
Mar. 1	II	1960	1014·5	336	1·15	0·98	0·17	30·37	0·37	1·12	0	0·46	74·3	7.30 a.m.
				308	1·13	1·10	0·06	31·69	0·34	1·12		0·51		
2	II + 2 gms. Na_2HPO_4 ...	1795	1015·5	440	1·27	1·40	0·0	30·45	0·43	1·30	0	0·44	74·3	
3	II + 3 ,, ,, ...	1765	1015	392	1·52	1·42	0·10	30·66	0·39	1·21	0	0·47	74·3	
4	II + 3 ,, ,, ...	2035	1013	374	1·89	1·28	0·11	30·83	0·37	1·21	0	0·47	74·2	7.15 a.m.
				402	1·39	1·37	0·07	30·65	0·40	1·24		0·46		
5	II + 15 c.c. glycerophosphoric acid ...	1722	1014	504	2·10	2·13	0·0	27·02	0·38	1·21	0	0·45	74·2	III, 3 times.

Mar.	6	II + 10 c.c. glycerophosphoric acid	...	—	—	—	—	—	—	—	—	—	—	—	—	
	7	—		—	—	—	—	—	—	—	—	—	—	—	—	
	8	—		—	—	—	—	—	—	—	—	—	—	72·6	1 stool.
	9	—		—	—	—	—	—	—	—	—	—	—	73·2	
	10	Mixed	...	710	1024	576	2·23	2·02	0·21	25·21	0·67	1·30	0	0·86	73·2	
	11	Mixed	...	1220	1020	544	1·90	1·94	0·0	28·26	0·71	1·36	0	0·83	—	
	12	Mixed	...	1620	1017	520	2·16	2·02	0·14	27·79	0·79	1·03	0	0·82	—	
	13	Mixed	...	1850	1017	496	1·98	1·96	0·02	25·54	0·63	1·04	0	0·80	—	
	14	Mixed	...	980	1026	568	2·18	2·27	0·0	21·82	0·62	1·05	0	0·88	—	
	15	Mixed	...	1880	1017	520	1·88	1·84	0·04	30·16	0·64	0·98	0	0·89	—	9 p.m.
						537	2·06	2·01	0·07	26·46	0·68	1·13		0·85		
	16	II + 2 gms. $CaCl_2$	...	1420	1014	360	0·94	0·91	0·03	29·84	0·67	0·95	0	0·62	73·8	8.0 a.m.
	17	II + 4 ,, ,, ...	...	1980	1012	296	0·56	0·49	0·07	28·78	0·62	1·00	0	0·46	73·5	
	18	II + 4 ,, ,, ...	...	1670	1014·5	336	0·68	0·51	0·17	27·90	0·65	0·97	0	0·42	73·3	7.45 a.m.
						331	0·73	0·64	0·09	28·83	0·65	0·97		0·50		
	19	II + 8 gms. Ca-glycerophosphate	...	1680	1015	496	1·83	1·62	0·21	29·98	0·61	0·93	0	0·41	73·4	
	20	II + 8 ,, ,,	...	1790	1013	520	2·16	2·08	0·08	29·82	0·61	0·95	0	0·43	73·4	10.15 p.m.
	21	II + 8 ,, ,,	...	1640	1015	488	2·03	2·00	0·03	29·13	0·49	0·98	0	0·42	73·3	
						501	2·01	1·90	0·11	29·63	0·57	0·95		0·42		
	22	II + 15 gms. lecithin	...	1800	1015	392	1·75	1·74	0·01	28·08	0·51	1·17	0	0·47	73·3	
	23	II + 25 ,, ,,	...	1880	1013	424	2·13	2·05	0·08	28·60	0·63	1·07	0	0·44	73·3	7.30 a.m.
	24	II + 25 ,, ,,	...	1698	1014	504	2·31	2·25	0·06	27·36	0·69	1·07	0	0·41	73·4	
	25	II + 15 ,, ,,	...	1810	1013	464	2·03	1·93	0·10	28·82	0·77	1·07	0	0·45	73·2	6.30 a.m.
						446	2·05	1·99	0·06	28·22	0·62	1·08		0·44		
	26	II + 5 gms. Na-phosphate + 8 gms. Ca-lactate		2112	1012·5	355	1·53	1·25	0·28	28·37	0·63	1·07	0	0·42	72·9	
	27	II + 5 ,, ,, + 8 ,, ,,		1990	1014	336	1·37	1·44	0·0	29·22	0·50	1·06	0	0·41	72·5	
	28	II + 5 ,, ,, + 8 ,, ,,		1820	1014·5	272	1·50	1·51	0·0	29·67	0·36	1·06	0	0·45	72·3	7.30 a.m.
						321	1·47	1·40	0·09	29·07	0·50	1·06		0·43		

TABLE I (*continued*).

Date	Diet	Volume	Sp. gr.	Acidity N/10	Total P_2O_5, gm.	Inorg. P_2O_5, gm.	Org. P_2O_5, gm.	Urea, gm.	Ammonia, gm.	Creatinine, gm.	Creatine, gm.	Uric acid, gm.	Body weight, Kilogm.	Stools passed
Mar. 29	II + 5gms. Na-phosphate + 3gms. nucleic acid	1855	1014	288	1·83	1·84	0·0	26·64	0·30	1·08	0	0·46	72·4	
30	II + 5 ,, ,, + 5 ,, ,,	1760	1014·5	256	1·77	1·63	0·14	29·04	0·33	1·08	0	0·48	72·3	7.30 a.m.
31	II + 5 ,, ,, + 7 ,, ,,	1650	1016	328	1·85	1·81	0·04	28·40	0·36	1·07	0	0·47	72·3	7.30 a.m. & 10.30 p.m.
				291	1·82	1·76	0·06	28·09	0·33	1·06		0·47		
Apr. 1	II + 5 gms. Na-phosphate + 1 gm. creatine	1650	1015	336	1·60	1·52	0·08	26·05	0·44	1·12	0	0·44	72·4	
2	II + 5 ,, ,, + 2 ,, ,, + 1 gm. $CaCl_2$	1860	1013·5	296	1·50	1·42	0·08	26·28	0·48	1·06	0	0·44	72·2	7.15 a.m.
				316	1·55	1·47	0·08	26·17	0·46	1·09		0·44		
3	II (rice) + 5 gms. Na-phos. + 3 gms. creatine	2158	1011·5	371	1·61	1·58	0·03	26·92	0·59	1·13	0·33	0·42	71·8	
4	II (rice) + 5 ,, ,, + 2·5 ,, ,,	1785	1014	368	1·57	1·48	0·09	26·14	0·62	1·12	0·11	0·43	72·0	
5	II (rice) + 5 ,, ,, ...	1550	1015	361	1·60	1·66	0·0	26·62	0·54	1·01	0	0·45	—	4.0 p.m.
				367	1·59	1·51	0·04	26·59	0·58	1·09		0·43		
6	II + 5 gms. Na-phosphate	1920	1014·5	336	1·52	1·45	0·07	28·85	0·65	1·09	0	0·46	71·7	
7	II + 5 gms. Na-phosphate + 0·2 gms. xanthine	2170	1012	330	1·46	1·38	0·08	26·94	0·56	1·13	0	0·50	71·5	
8	II + 5 ,, ,, + 1 ,, ,, + 1 gm. $CaCl_2$	1775	1014	280	1·40	1·34	0·06	27·68	0·48	1·06	0	0·52	71·5	7.30 a.m.
				305	1·43	1·36	0·07	27·31	0·52	1·09		0·51		
9	II + 3 gms. Na-phosphate + 0·5 gms. guanine	1860	1014	320	1·67	1·63	0·04	27·97	0·49	1·07	0	0·56	—	
10	II + 5 ,, ,, + 2·0 ,, ,,	1775	1013	344	1·60	1·51	0·09	27·18	0·44	1·09	0	0·58	71·2	7.30 a.m.
				332	1·63	1·57	0·06	27·58	0·47	1·08		0·57		
11	II + 5 gms. Na-phosphate	1780	1014	344	1·75	1·67	0·08	29·87	0·51	1·11	0	0·63	71·2	7.30 a.m.

Apr.	12	II + 2 gms. Na-phosphate + 12 gms. lemco ...	1540	1015	336	1·77	1·80	0·0	28·93	0·44	1·31	0	0·59	71·4	
	13	II + 24 gms. lemco	2060	1016	361	2·10	2·05	0·05	33·56	0·49	1·68	0	0·75	71·4	7.30 a.m.
	14	II + 48 gms. ,, ...	1800	1017·5	456	2·89	2·78	0·11	32·87	0·48	2·08	0	0·95	71·5	
					384	2·25	2·21	0·05	31·79	0·47	1·69		0·76		
	15	II + 2·5 gms. Na-phosphate	2135	1013	317	1·76	1·68	0·08	30·77	0·58	1·15	0	0·73	—	7.30 a.m. &
	16	II + 4 gms. arabinose	1720	1014	304	1·62	1·66	0·0	27·77	0·46	1·09	0	0·52	71·0	10.30 p.m.
					311	1·69	1·67	0·04	29·27	0·52	1·12		0·62		
	17	100 gms. egg-white + 250 gms. boiled out meat + 5 gms. Na-phosphate	1330	1016	376	2·08	1·91	0·17	26·40	0·41	1·08	0	0·54	70·9	
	18	250 gms. boiled out meat + 2·5 gms. Na-phosphate	1300	1016	376	2·05	2·08	0·0	27·17	0·45	1·10	0	0·58	70·5	7.30 a.m.
					376	2·07	1·99	0·08	26·78	0·43	1·09		0·56		
	19	II + 5 gms. Na-phosphate	1530	1015	360	1·93	1·91	0·02	27·85	0·46	1·09	0	0·56	70·5	7.30 a.m.
	20	400 gms. egg-white + 250 gms. herring roe + 2·5 gms. Na-phosphate	1370	1020	504	2·66	2·64	0·02	27·66	0·61	1·19	0	1·01	71·2	7.30 a.m. & 10.45 p.m.
	21	500 gms. herring roe	1600	1021·5	822	4·88	4·85	0·03	31·42	0·72	1·32	0	1·06	70·6	3.30 p.m.
	22	500 ,, ,, ...	1940	1020	1008	5·78	5·65	0·13	28·48	1·26	1·30	0	1·42	70·9	7.30 p.m.
					778	4·44	4·38	0·06	29·19	0·86	1·27		1·16		
	23	Mixed	2230	1015	883	4·72	4·65	0·07	29·03	0·99	1·14	0·11	1·25	70·7	7.45 a.m.
	24	Mixed	2313	1012	653	2·92	2·86	0·06	30·29	1·17	1·40	0	0·97	—	
					768	3·82	3·75	0·07	29·66	1·08	1·27		1·11		

TABLE II.

Nitrogen Values.

Date	Total N.	Urea N.	Creatinine N.	Creatine N.	N. in Uran. Acet.	Purine Base N.	Uric Acid N.
Feb. 13	14·78	—	0·49	—	0·17	0·06	0·28
14	16·47	14·21	0·55	—	0·09	0·05	0·33
15	13·38	11·59	0·49	—	0·09	0·04	0·23
16	16·13	14·04	0·53	—	0·07	0·07	0·26
17	16·91	14·58	0·58	—	0·02	0·06	0·37
18	17·75	15·15	0·48	—	—	0·06	0·29
	16·01	13·91	0·52		0·09	0·06	0·29
19	17·81	14·9†	0·52	—	0·04	0·04	0·28
20	17·25	14·82	0·46	0·07	0·05	0·07	0·29
21	16·48	14·58	0·47	0·07	0·05	0·04	0·27
22	17·42	15·50	0·50	0·02	0·09	0·05	0·25
23	16·27	14·12	0·57	0·09	0·05	0·04	0·25
24	16·30	14·11	0·50	0·07	0·09	0·03	0·25
25	18·09	15·96	0·51	0·05	0·09	0·04	0·25
	17·09	15·01	0·50		0·07	0·04	0·26
26	16·13	14·69	0·41	—	0·03	0·04	0·19
27	16·57	15·10	0·39	—	0·02	0·03	0·18
28	16·46	15·06	0·46	—	0·03	0·04	0·16
March 1	15·74	14·17	0·42	—	0·04	0·02	0·15
	16·23	14·75	0·42		0·03	0·03	0·17
2	15·57	14·21	0·49	—	0·03	0·03	0·15
3	15·90	14·31	0·45	—	0·03	0·02	0·16
4	16·47	14·39	0·45	—	0·03	0·02	0·16
	15·91	14·30	0·46		0·03	0·02	0·16
5	14·56	12·61	0·45	—	0·02	0·02	0·15
6	—	—	—	—	—	—	—
7	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—

TABLE III.

In per cent. of Total Nitrogen.

Urea	Ammonia	Creatinine	Creatine	Uran. Acet.	Purine	Uric Acid	Undetermined
—	3·48	3·41	—	0·72	0·4	1·97	—
86·3	3·07	3·32	—	0·59	0·33	2·0	4·39
85·7	3·59	3·61	—	0·69	0·30	1·74	3·61
87·0	3·22	3·27	—	0·6	0·43	1·58	2·75
86·3	3·20	3·41	—	0·11	0·34	2·17	4·57
85·8	3·0	2·71	—	—	0·34	1·61	5·54
84·2	2·8	2·95	—	0·22	0·23	1·57	7·37
85·93	2·6	2·65	0·43	0·29	0·38	1·7	5·76
85·53	2·79	2·95	0·44	0·33	0·26	1·62	2·58
89·98	2·25	2·9	0·1	0·44	0·27	1·49	3·57
87·0	2·38	3·53	0·55	0·27	0·26	1·56	4·55
86·67	2·48	3·1	0·43	0·54	0·17	1·55	5·06
88·1	2·29	2·85	0·28	0·52	0·21	1·38	4·35
91·08	1·96	2·52	—	0·17	0·24	1·16	2·87
91·15	1·96	2·3	—	0·11	0·18	1·11	2·29
91·48	2·09	2·8	—	0·17	0·22	0·97	2·27
90·44	2·07	2·67	—	0·2	0·15	0·97	3·50
90·2	2·85	3·11	—	0·17	0·22	0·94	1·51
90·0	2·46	2·85	—	0·19	0·15	0·98	3·37
87·0	2·25	2·75	—	0·18	0·14	0·95	6·73
86·6	2·78	3·11	—	1·0	0·18	1·03	5·35
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—

March	10	14·22	11·77	0·49	—	0·01	0·06	0·19	82·73	4·69	3·42	—	0·09	0·44	1·81	6·32
	11	15·06	13·19	0·51	—	0·01	0·04	0·18	87·59	4·79	3·39	—	0·07	0·27	1·17	2·72
	12	14·90	12·97	0·35	—	0·02	0·03	0·18	87·02	5·22	2·34	—	0·12	0·18	1·19	3·93
	13	13·66	11·92	0·39	—	0·02	0·06	0·17	87·26	4·5	2·85	—	0·15	0·42	1·22	3·6
	14	12·21	10·61	0·39	—	0·02	0·03	0·19	86·86	5·1	3·22	—	0·19	0·26	1·25	4·48
	15	15·68	14·07	0·37	—	0·03	0·03	0·20	89·73	4·09	2·35	—	0·21	0·21	1·59	1·82
		14·29	12·42	0·42		0·02	0·04	0·18								
	16	15·46	13·93	0·36	—	0·03	0·03	0·21	90·09	3·55	2·31	—	0·16	0·17	1·34	2·38
	17	15·23	13·43	0·38	—	0·02	0·03	0·15	88·21	3·35	2·4	—	0·13	0·21	1·01	4·69
	18	14·62	13·02	0·36	—	0·02	0·03	0·14	89·0	3·65	2·62	—	0·09	0·18	0·96	3·50
		15·10	13·46	0·37		0·02	0·03	0·17								
	19	15·40	13·99	0·35	—	0·02	0·02	0·14	90·83	3·24	2·25	—	0·09	0·15	0·89	2·55
	20	15·90	13·92	0·36	—	0·02	0·03	0·14	87·54	3·16	2·21	—	0·09	0·20	0·90	5·67
	21	15·06	13·59	0·37	—	0·02	0·03	0·14	90·08	2·72	2·44	—	0·09	0·17	0·93	3·62
		15·45	13·83	0·36		0·02	0·03	0·14								
	22	14·78	13·10	0·44	—	0·03	0·02	0·16	88·6	2·83	2·95	—	0·2	0·14	1·06	4·22
	23	14·90	13·31	0·40	—	0·01	0·02	0·15	89·36	3·4	2·86	—	0·07	0·16	0·99	3·78
	24	14·17	12·76	0·40	—	0·02	0·02	0·14	90·05	3·99	2·83	—	0·12	0·17	0·96	1·88
	25	15·34	13·45	0·40	—	0·02	0·02	0·15	87·69	4·13	2·62	—	0·10	0·16	1·00	4·3
		14·80	13·15	0·41		0·02	0·02	0·15								
	26	14·78	13·24	0·40	—	0·02	0·02	0·14	89·7	3·39	2·72	—	0·11	0·16	0·95	2·99
	27	14·90	13·63	0·40	—	0·02	0·02	0·14	91·4	2·78	2·67	—	0·11	0·15	0·92	1·9
	28	15·68	13·85	0·40	—	0·02	0·02	0·15	88·3	1·9	2·6	—	0·11	0·12	0·96	6·3
		15·12	13·57	0·40		0·02	0·02	0·14								
	29	14·56	12·52	0·41	—	0·01	0·02	0·15	86·71	2·06	2·78	—	0·09	0·12	1·05	7·2
	30	14·62	13·55	0·39	—	0·02	0·02	0·16	92·69	1·86	2·64	—	0·11	0·14	1·09	1·47
	31	14·84	12·98	0·40	—	0·02	0·02	0·16	87·44	2·0	2·7	—	0·14	0·14	1·06	6·52
		14·67	13·02	0·40		0·02	0·02	0·16								
April	1	13·83	12·16	0·42	—	0·02	0·02	0·15	87·89	2·74	3·05	—	0·13	0·15	1·06	4·9
	2	13·55	12·26	0·40	—	0·02	0·02	0·15	90·51	3·51	2·95	—	0·12	0·13	1·09	1·69
		13·69	12·21	0·41		0·02	0·02	0·15								

TABLE II (*continued*).

Nitrogen Values.

Date	Total N.	Urea N.	Creatinine N.	Creatine N.	N. in Uran. Acet.	Purine Base N.	Uric Acid N.
April 3	14·14	12·56	0·43	0·10	0·02	0·02	0·14
4	14·06	12·30	0·42	0·08	0·02	0·02	0·14
5	14·20	12·43	0·38	—	0·02	0·02	0·15
	14·13	12·40	0·41		0·02	0·02	0·14
6	15·06	13·47	0·41	—	0·02	0·02	0·16
7	14·22	12·57	0·43	—	0·02	0·03	0·17
8	14·50	12·92	0·40	—	0·02	0·03	0·17
	14·39	12·75	0·41		0·02	0·03	0·17
9	14·95	13·13	0·40	—	0·02	0·02	0·19
10	14·22	12·68	0·41	—	0·03	0·03	0·19
	14·59	12·91	0·40		0·02	0·02	0·19
11	15·18	13·94	0·42		0·02	0·02	0·21
12	14·84	13·40	0·49	—	0·02	0·02	0·20
13	17·58	15·66	0·63	—	0·02	0·03	0·25
14	17·25	15·34	0·78	—	0·02	0·03	0·32
	16·56	14·80	0·63		0·02	0·03	0·26
15	16·20	14·36	0·43	—	0·02	0·03	0·24
16	14·22	12·97	0·41	—	0·02	0·02	0·17
	15·21	13·67	0·42		0·02	0·03	0·20
17	14·00	12·32	0·41	—	0·02	0·03	0·18
18	14·00	12·68	0·41	—	0·02	0·03	0·19
	14·00	12·50	0·41		0·02	0·03	0·18
19	14·73	13·00	0·41		0·03	0·03	0·19
20	15·23	12·91	0·45	—	0·02	0·02	0·37
21	17·03	14·66	0·49	—	0·02	0·03	0·39
22	17·32	13·29	0·49	—	—	0·04	0·47
	16·53	13·62	0·48		0·02	0·03	0·41
23	15·91	13·55	0·43	0·04	0·02	0·04	0·40
24	16·88	14·00	0·50	—	0·01	0·05	0·32
	16·39	13·77	0·46		0·02	0·04	0·36

TABLE III (*continued*).

In per cent. of Total Nitrogen.

Urea	Ammonia	Creatinine	Creatine	Uran. Acet.	Purine	Uric Acid	Undetermined
88·85	3·45	3·01	0·75	0·15	0·15	0·99	2·65
86·76	3·65	3·00	0·25	0·14	0·16	1·02	5·02
87·5	3·87	2·69	—	0·13	0·16	1·06	4·59
89·77	3·57	2·73	—	0·14	0·14	1·03	2·62
88·42	3·23	2·99	—	0·13	0·21	1·17	3·85
89·8	2·7	2·75	—	0·11	0·19	1·19	3·26
89·14	2·69	2·69	—	0·15	0·16	1·25	3·92
89·19	2·56	2·87	—	0·17	0·16	1·36	3·67
91·77	2·79	2·76	—	0·13	0·14	1·38	1·08
91·66	2·43	3·3	—	0·11	0·16	1·33	1·01
89·09	2·29	3·58	—	0·1	0·15	1·48	3·31
88·8	2·3	4·52	—	0·09	0·15	1·84	2·3
88·02	2·92	2·66	—	0·11	0·19	1·5	4·71
91·43	2·59	2·87	—	0·15	0·15	1·22	1·59
88·0	2·4	2·93	—	0·11	0·19	1·29	5·08
90·58	2·62	2·97	—	0·13	0·18	1·38	2·14
88·2	2·43	2·79	—	0·09	0·2	1·27	5·42
84·75	3·31	2·93	—	1·01	0·17	2·43	5·41
86·09	3·46	2·9	—	1·3	0·17	2·27	3·81
76·73	5·87	2·81	—	3·16	0·26	2·73	8·44
85·16	5·1	2·68	0·21	0·1	0·25	2·52	3·98
82·92	5·67	2·96	—	0·06	0·3	1·91	7·18

Diet III.

Herring roe	100	+	200	+	200	=	500 gm.	=	100	—	—
Potato	200	+	300	+	300	=	800 ,,	=	16	160	—
Butter	80	+	50	+	50	=	130 ,,	=	—	—	104
Sugar	20	+	20	+	20+20	=	80 ,,	=	—	80	—
									116	240	104

In addition a constant amount of water (1000 c.c.) was taken daily mixed with whisky in portions of 25 c.c.: only on certain days when various compounds were administered was the quantity greater and it then amounted to an extra 50 or 100 c.c. 2 gm. of NaCl were also taken daily.

The quantities of each of these foodstuffs were most carefully weighed out though an examination of the total nitrogen appearing in the urine does not appear to justify the extreme care employed. The variations are most probably due to variations in the water content of the egg-whites of which some 25 to 30 were required daily, but they may be partly due to another factor which has been noticed during the course of the experiment (see under Total Nitrogen results).

The experiment was commenced by estimating the output of the various constituents on an ordinary mixed diet for one week so that the intake of protein during the periods of the selected diets should correspond as nearly as possible with the amount taken during this period, and as will be seen from the table the excretion of total nitrogen is almost the same throughout. The beefsteak diet was first administered; this was followed by the egg-white diet. After one week the subject unfortunately suffered from an attack of influenza so that the experiment had to be discontinued for about 10 days; analyses of the urine were, however, continued except for a period of 4 days. The egg-white diet was then again taken for a period of 34 days and during this time the effect of the addition of various compounds was examined. Excepting the first 3 days at the commencement of the egg-white diet and when calcium glycerophosphate and lemco were given 3 gm. of sodium phosphate were added to the diet since it was considered that phosphorus was an essential constituent of the diet (egg-white contains only traces of phosphorus, 0·1 % in the dry substance): a dose of calcium chloride was given at the commencement of the second period of egg-white so that the effect of glycerophosphoric acid by itself might be ascertained. It was found that the addition of lemco to this diet increased the output of uric acid and for 2 days during this period a protein diet (= 110 gm.) consisting of boiled out meat was given instead of egg-white. For a short period also a change in the carbohydrate

constituent was made, rice being substituted for potato. The herring roe diet concluded the experiment; instead of a week as originally intended this diet was taken only for 3 days as its effect was such that the subject felt as if he had emerged from a severe illness and it is doubtful whether a week on this diet would have been possible; the effect may, however, be largely due to fatigue as the subject worked daily in the laboratory throughout the experiment as well as attending to his diet.

The results of the experiment with the details of the additions to the diet are contained in the preceding tables, the last two of which give the nitrogen of the nitrogenous constituents and the calculation of these in percentages of the total nitrogen.

In the estimation of the various constituents—all carried out in duplicate—the methods of analysis adopted were:

Total nitrogen by Kjeldahl's method; acidity, ammonia, urea, creatinine and creatine by Folin's methods; uric acid by the Folin-Schaffer method; purine bases by the Camerer-Arnstein method in the filtrate from the ammonium urate using 400 c.c. = 261 c.c. urine. (Cf. Kennaway, Leathes and Cathcart[4], Kennaway[5].) The amount of nitrogen in the uranium acetate precipitate obtained in the Folin-Schaffer method was also estimated, the precipitate being thoroughly washed for 3 days with water, then boiled with magnesium oxide to remove the last traces of ammonia and the nitrogen estimated by Kjeldahl's method.

The total phosphorus in the urine was estimated by Neumann's method as modified by Plimmer and Bayliss and inorganic phosphates by precipitation with ammonium magnesium citrate (cf. Mathison[6]).

In addition to these analyses the number of leucocytes in the blood were kindly counted for us by Dr Thiele to whom we express our best thanks. This seemed to us very necessary since Horbaczewski showed that a definite relationship existed between the number of leucocytes in the blood and the output of uric acid and especially as most of the later investigators neglected this detail. The leucocyte count was:

Date	No.	Sl.	Ll.	H.	N.	O.	R.	Diet
Feb. 13	16,000	22	17	1	58	2	0	Mixed.
15	19,000	23	18	1	56·2	1·8	0	,,
20	17,500	19	14	1	63	2·4	0·6	Meat.
22	19,000	16	15	1	68	2·0	0	,,
24	22,000	20	14	2	62	1·8	0·2	,,
26	18,000	22	16	2	58·8	1·2	0	Egg-white.
Mar. 1	12,000	22	14	3	61	0	0	,,
3	7,000	31	5	0·8	62	1·2		,,
5	4,200	25·8	12	2	59·2	1		Egg-white + glycerophosphoric acid.
8	10,500	22			78	0	0	Mixed.
10	15,000	16	12	1	70	1	0	,,
15	12,000	18	12	2	67	1	0	Egg-white + $CaCl_2$.
17	10,000	19	12	1	68	0	0	,, ,,
19	7,000	21	14	2	62	1	0	Egg-white + Ca-glycerophosphate.
20	7,600							,, ,,
22	10,000	18	12	2	67	1	0	Egg-white + lecithin.
24	6,500	21	18	2	58	1	0	,, ,,
26	12,000	17	14	3	66	0	0	Egg-white + Ca lactate.
29	10,500	19	16	2	62	1	0	Egg-white + nucleic acid.
30	5,200	0	48	0	44	2	0	,, ,,
31	6,000	10	44	0	45	1	0	Egg-white + creatine.
Apr. 1	11,000	16	24	1	58	1	0	,, ,,
2	9,500	20	21	1	56	2	0	Egg-white, rice + creatine.
3	13,500	20	14	0	65	1	0	,, ,,
5	13,000							Egg-white.
8	13,000							Egg-white + xanthine.
10	7,000							Egg-white + guanine.
13	15,000	20	8	2	66	4	0	Egg-white + lemco.
14	17,000							Egg-white + lemco.
15	10,000	35	8	1	56	0	0	Egg-white + arabinose.
16	8,000							Egg-white + boiled out meat.
17	7,000		37		63			Boiled out meat.
19	7,800		47		53			Egg-white + herring roe.
20	7,500		39		61			Herring roe.
21	8,500		28	11	59	1	0	,, ,,
22	11,800	19	10	1	68	2	0	Mixed.
23	11,700	19	9	1	70	1	0	,,

From which it will be noticed that the normal number of leucocytes in the subject's food is high: it may be accounted for by his long residence in India and from his having suffered from malaria.

Remarks.

1. *Effect of Diet.* That no ill-effect resulted from the egg-white diet upon which the subject lived for 4½ weeks is shown by the small loss of body weight which occurred. His initial weight of 76 kilos. dropped to 73 kilos. after the attack of influenza, but from that time to the end of the experiment there was a loss of only 2 kilos., the subject's final weight being about 71 kilos.

2. *Volume of Urine.* Although the subject took practically the same quantity of water with his food daily, there was a diurnal variation in the volume of urine, which is without doubt attributable to climatic conditions.

3. *Acidity.* Very great changes are noticeable in the acidity of the urine on the various diets: on the mixed diet it was distinctly higher than on the meat diet or the egg-white diet; on the herring roe diet the acidity was extraordinarily high. On the whole, the acidity corresponds with the output of phosphates. It was lowest on the pure egg-white diet and it rose slightly when sodium phosphate (Mar. 2—4) was given; there was a distinct increase when calcium glycerophosphate and lecithin were taken which was not maintained by calcium lactate. Nucleic acid and the purines had no effect, but creatine produced a distinct, although small, rise. Lemco also caused an increase in the acidity and this is probably due to the increased amount of phosphates.

4. *Total* P_2O_5 *and Inorganic* P_2O_5. Since inorganic P_2O_5 constitutes 90—100 per cent. of the total P_2O_5 a rise in the output of inorganic P_2O_5 accounts for the whole change observable in the amount of the total P_2O_5. The quantity excreted depends entirely on the intake of P_2O_5; on the pure egg-white (P_2O_5-free) the output was about 1 gm. per day but as this was not continued for a sufficiently long time no conclusions can be drawn.

5. *Organic* P_2O_5. It was hoped that some knowledge of the origin and nature of the organic P_2O_5 in the urine might be obtained during this experiment, but the daily output in our subject was extremely irregular; on some days no organic P_2O_5 was excreted and then on the following day a double quantity would appear in the urine. The average amounts on the various diets were practically the same and the

addition of the various substances to the diet made no difference either in its amount or in the curious manner in which it was excreted. The organic P_2O_5 in the urine is therefore uninfluenced by diet and must originate in the body, *i.e.* it must be endogenous.

6. *Total Nitrogen.* Throughout the various periods of the experiment the total nitrogen in the urine was only fairly constant: it was lowest on the egg-white diet about 15 gm. per diem and highest on the meat diet about 17 gm. per diem; on the mixed diet first period it was 16 gm., second period 14 gm.; on the herring roe diet it rose to 17 gm. per diem. The rise when lemco was given must not be taken into account as no alteration in the intake of egg-white was made on these days to correspond with the amount of nitrogen contained in it.

It will be noticed from the figures that there was on each diet, especially with the egg-white one, on certain days a higher total nitrogen output than on the two or three days preceding followed by a lower output. This may be accounted for by slight changes in the intake of nitrogen due to differences in the amount of water in the meat or egg-white but it may be partly attributed to non-absorption of the diet when the bowels were not opened since on the days subsequent to the passing of stools the total nitrogen was always higher.

The relationship of total nitrogen in the urine and the opening of the bowels is particularly noticeable when, on glancing through the total nitrogen figures and noting a higher figure, a comparison is made with the time a stool was passed; on nine days a high total nitrogen output was found by one of us to coincide nearly exactly with the time the bowels were opened without reference to the table.

7. *Urea.* The excretion of urea runs, as would be expected, almost parallel with that of the total nitrogen throughout the experiment. The quantity excreted made up from 84 to 91 per cent. of the total nitrogen, the highest figures occurring on the pure egg-white diet.

8. *Ammonia.* The amount of ammonia present in the urine on the various diets varied considerably: it was highest on the mixed diet and lowest on the pure egg-white diet and varied with the nature of the substance added to this diet. The addition of sodium phosphate caused only a very slight increase in its amount and no increase was observed when, for one day, glycerophosphoric acid was given. The addition of calcium chloride to the egg-white diet kept the output of ammonia up to the same level as on the mixed diet; calcium glycerophosphate did likewise. When lecithin was taken the amount of

ammonia was slightly greater and it diminished with calcium lactate. Nucleic acid produced no increase. Creatine produced a distinct rise which diminished subsequently and another increase was only noticed with the herring-roe diet.

9. *Creatinine.* The output of creatinine was distinctly lower on the egg-white diet in the first period when this was taken than on the mixed and meat diets and is most probably accounted for by the presence of small quantities of creatinine in these diets. During the second mixed diet a fall in the excretion took place, but this is most probably due to a new lot of picric acid solution which was prepared from material from another source. The output was subsequently nearly constant except when lemco was given and as this contained creatinine (1 gm. = 0·0295 gm. creatinine) the increase was due to its presence: the extra output averaged 75 per cent. of that ingested, a figure which agrees closely with that of previous observers (80 per cent.).

10. *Creatine.* Creatine was only present in the urine during the meat diet, when creatine was given and on the first day of the mixed diet at the end of the experiment. Not until 2·5 gm. of creatine were given did it appear in the urine and when this amount was reduced it disappeared. The quantity then found in the urine was roughly the same as that excreted on the meat diet. From this result an approximate estimation of the amount of creatine in meat may be made: when 2·5 gm. of creatine were added to the diet it appeared in the urine but it was absent when 2 gm. were taken; hence 500 gm. meat will contain about 2·5 gm. of creatine.

The administration of creatine to the egg-white diet produced no increase in the amount of creatinine in the urine, the highest creatinine figure 1·13, though higher than on the previous day, being reached on other occasions.

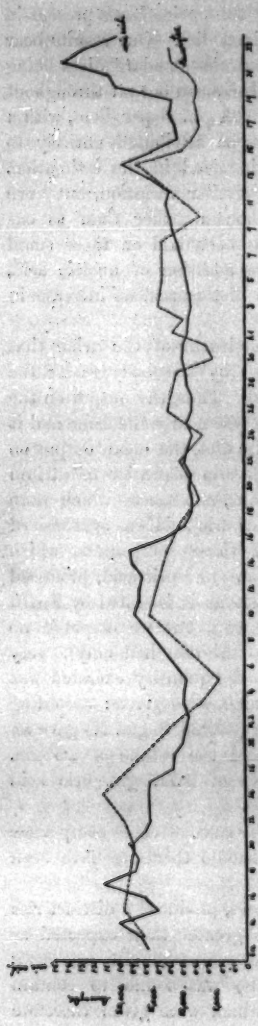
11. *Nitrogen in the Uranium acetate Precipitate.* So far as we know no estimations of the amount of nitrogen precipitated by the uranium acetate employed in the Folin-Schaffer uric acid method have been made: it was therefore of interest to make such estimations. The amount of nitrogen in the precipitate was very small: it was highest on the mixed and meat diets and the low figure when once obtained on the egg-white diet remained constant throughout the experiment. It is probable that some of the nitrogen in this precipitate represents a portion of the purine base nitrogen, as Kennaway has informed us that xanthine is partially thrown out of solution by the reagent.

12. *Purine Bases.* The greater part of the purine bases present in urine arise from the tea and coffee of a mixed diet. Our results bear out this statement, the amount excreted on the standard diets being considerably less than on the mixed diet. The mean output throughout the experiment on the standard diets was 0·02 gm. N per diem, with a variation of 0·01 gm. Such a figure must be attributed entirely to experimental error in the manipulations involved in the estimation. Previous investigators have also observed a similar variation, but have attributed it to actual changes in metabolism rather than to experimental error, and too much stress has been laid on these small variations. It should be noticed that the addition of nucleic acid, guanine, xanthine and also the herring roe diet caused no increase in the output of purine bases.

13. *Uric Acid.* It is only in this constituent of the urine that marked variations in the amount excreted by the body under the several conditions of diet have been observed. Thus, the output on the mixed (the two periods) and the meat diets was nearly the same and it decreased to almost one-half on the egg-white diet, the mean output on this diet being about 0·43 gm. per day. The administration of calcium lactate, calcium glycerophosphate and lecithin, substances which were given for the purpose of determining whether uric acid was synthesised in the body from ammonia or urea and these compounds, which contain a straight chain of three carbon atoms like uric acid, produced no increase. Rice was substituted for potato as it is stated by Folin that potato increases the output of uric acid, but we observed no difference. The addition of nucleic acid to the diet had only a very slight effect on the output of uric acid, but the quantity excreted was distinctly greater when xanthine and guanine were given: according to our results the intake of 1·9 gm. xanthine (= 0·6988 gm. N) gave an increase of 0·057 gm. uric acid nitrogen and the intake of 2·5 gm. guanine (= 1·159 gm. N) gave an increase of 0·097 gm. uric acid nitrogen.

Former observers have found that 50 per cent. of these compounds was excreted as uric acid and the increase should therefore have been 0·35 gm. N and 0·70 gm. N respectively.

Lemco, which was given in increasing doses, produced a distinct rise in the output of uric acid. This rise was greater than expected as more than half of the purine nitrogen was converted into uric acid: 1 gm. lemco (before and after hydrolysis) was found to contain 0·0063 gm. purine nitrogen; the 72 gm. which were given therefore



contained 0·5292 gm. purine nitrogen; if half of this had been converted into uric acid the rise should have been 0·2646 gm. N instead of which a rise of 0·33 gm. N was observed.

In meat therefore a substance is present which increases the output of uric acid; it is present in the extractives as boiled out meat produced no rise: it is probably not a purine nor a pentose, the other constituent of nucleic acid, as arabinose had no effect on the uric acid output.

The greatest output of uric acid occurred on the herring roe diet. The quantity excreted was however much smaller than expected if 50 per cent. of the purines in herring roe be converted into uric acid: 1 gm. herring roe, when hydrolysed, contained 0·005786 gm. purine nitrogen; the intake of purine nitrogen was therefore 7·2325 gm.; 0·80 gm. N were found as uric acid in the urine instead of 3·6163 gm.

14. *Leucocytes.* The large number of leucocytes in the blood on the mixed and meat diets gradually decreased on the egg-white diet to about half the original number, from 18,000 to 7,000 per cmm.; the very low figure of 4,200 was found just previous to the attack of influenza. The same decrease occurred again when the egg-white diet was taken after the attack of influenza the figures being 15,000 on the mixed diet and about 7,000 on the egg-white diet. Lecithin, when added to the egg-white, increased the number slightly; but the average number

remained about 7,000. A higher number was present when the calcium lactate was given; and this number decreased when nucleic acid was taken and rose slightly on the rice and creatine diet. The addition of xanthine and guanine made no difference to this number and there was subsequently a fall to 7,000, the mean figure on the egg-white diet. The number of leucocytes in the blood was doubled when lemco was given, and the level of 7,000 was again reached three days subsequently on the boiled out meat diet. The herring roe diet increased the number of leucocytes in the blood slightly and the number rose to about 12,000 on the final days of the experiment (mixed diet). On the meat diet therefore the number of leucocytes in the blood was twice as great as on the egg-white diet; on the herring roe diet the number was also greater. Excepting lemco, no great increase in the number was produced by the various compounds administered: the addition of lemco doubled the number of leucocytes in the blood.

15. *Relation of leucocytes to uric acid.* On comparing the number of leucocytes in the blood with the uric acid output it will be observed that these factors run parallel to one another. The uric acid output was high when the leucocyte content of the blood was high. Disappearance of leucocytes from the blood was associated with a fall in the output of uric acid. Thus, when 0·8 gm. uric acid was excreted (mixed and meat diets) the number of leucocytes in the blood was about 18,000 and when 0·43 gm. was excreted (egg-white diet) the number was 7,000. After the attack of influenza the figures were 0·8 gm. uric acid and 15,000 leucocytes; these became 0·43 gm. uric acid and 7,000 leucocytes. The output of uric acid during the period when calcium lactate, nucleic acid, rice and creatine were taken remained constant, but the number of leucocytes in the blood varied considerably; the mean figures, however, corresponded fairly closely with 0·44 gm. uric acid and 7,000 leucocytes. When the purines were given the leucocytes numbered 13,000 and the uric acid output was 0·6 gm. The relationship is very obvious during the lemco period, for then the leucocytes numbered 15,000 and the uric acid excreted was 0·95 gm., *i.e.* both figures about double those attained during the egg-white period. A decrease to 8,000 leucocytes and 0·55 gm. uric acid followed. No relationship was observed during the herring roe diet, but subsequently there were 13,000 leucocytes and 0·9 gm. uric acid. The curve graphically represents the statements made above.

Conclusions.

Although our results agree with those of previous investigators that the output of uric acid is greater on a meat or a mixed diet than on what has been termed a purine-free diet, yet they do not support the views which are now commonly held with regard to the origin of the uric acid in the urine. As a result of the work of Wiener and of Burian and Schur and others it is generally assumed that all the purines, whether endogenous or exogenous, undergo practically complete conversion into uric acid, and that one half of this was destroyed by oxidation whilst the other half appeared as uric acid. The fact that in our experiments the purine base output remained minimal and was not affected by any changes in the diet confirms the idea that these substances undergo total conversion in the body, but our results do not bear out the further statement that one half of the converted purines appears in the urine as uric acid. This is shown by the following facts:

1. Administration of guanine and xanthine gave practically no rise in the uric acid output.

2. Administration of very large quantities of purines in herring roe certainly increased the output of uric acid, but the excess of uric acid only corresponded to about one-tenth of the total purines ingested.

3. Liebig's extract of meat caused a great increase in the excretion of uric acid, more than half of the purines present in it being converted into uric acid. On the above view this could only be explained if it were assumed that with this substance for some reason or other uricolysis or destruction of uric acid had diminished.

There is thus scarcely any relation between purines and uric acid in our subject. Purines arising from the destruction of the nuclei of the leucocytes are therefore probably not the precursors of the uric acid in the urine as Horbaczewski supposed; if this were the case, then a decrease in the number of leucocytes should correspond with an increase in the uric acid output, but the reverse was the case. What then is the relationship between leucocytes and uric acid? In all living animals foodstuffs undergo metabolism: in mammals the end-product is urea, in birds, reptiles and fishes it is urea and uric acid, in many invertebrates it is uric acid. In leucocytes, as invertebrates, the end-product of metabolism is most probably uric acid. We are therefore inclined to conclude that the uric acid in the urine is an expression of their metabolism. Three times during our experiment a double amount of

uric acid was excreted when the number of leucocytes was double, and when the average number of leucocytes was greater, the output of uric acid was also greater pari-passu. Twice as many animals of the same weight and on the same diet would be expected to excrete twice as much. It may be objected that with the herring roe diet there was a large excretion of uric acid without any corresponding leucocytosis. The subject of the experiment, however, during this period felt extremely ill and we believe that there may have been during this time also an increase of leucocytes but that this was confined, as is known to be the case in typhoid fever, to the tissues of the intestinal wall. In typhoid there is an excessive production of uric acid which may be ascribed to the increase of leucocytes in the intestines. In pneumonia the increased output of uric acid runs parallel with leucocytosis of the blood; after the crisis, although a very large destruction of leucocytes is going on during the process of resolution in the lung tissue, the increased output of uric acid comes to an end.

The poisons produced in these diseases call forth a larger number of leucocytes into the blood to act as scavengers and this is probably the reason why the Liebig's extract of meat caused such a leucocytosis. It may be that some poison in it requires removal and a similar toxic state may be present in gout; for some reason or other a breakdown of muscle may occur with a corresponding liberation of poisonous extractives: leucocytosis then takes place and an increase in the quantity of uric acid follows. At least these hypotheses can reasonably be made from the above experiment.

Further work will be necessary before the view here put forward can be regarded as established, but our results certainly throw considerable doubt on the prevalent opinion that the purines of the food and of the tissues are the sole source of the uric acid in the urine.

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THE DISSOCIATION CURVE OF BLOOD.

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I. *Historical.*

The available information concerning the dissociation curves of the blood of different animals under varying conditions has been reviewed so frequently in late years that it is only necessary to refer the reader to such works as those of Bohr, of Loewy and of F. Müller in the text-books of Nagel and Oppenheimer.

From the general mass of information discussed in these articles a few points will be mentioned which will serve as an introduction to our own work.

(1) The dissociation curves of hæmoglobin solutions as determined by different observers, and indeed by the same observer (Hüfner) at different times, are not identical [1]. The general nature of the differences is shown by a consideration of the percentage saturation of hæmoglobin at two specified tensions of oxygen.

	Hüfner 'old curve'	Hüfner 'new curve'	Bohr [2]
Percentage saturation of oxygen at 14 mm. pressure	84	63	35
Percentage saturation of oxygen at 35 mm. pressure	93	80	65

(2) The dissociation curves for blood are no more uniform than those for hæmoglobin solutions. Loewy, for instance, working with human blood found the following values in different experiments:

Approximate tension (in mm.)	27	20	45
Percentage saturation in different experiments	49	41	59
	68	49	86
	—	58	—
	—	65	—

Whilst Zuntz and Loewy, working on dog's blood, arrived at the following, amongst other results:

Approximate tension (in mm.)	15	24	32
Percentage saturation in different experiments	12·5	42	54
	36	43	75
	54	63	—
	—	64	—

(3) Bohr discovered that the CO_2 tension had considerable effect on the percentage saturation of blood with oxygen. Probably some of the divergencies noted above are due to differences in the CO_2 tension.

(4) Even at constant CO_2 tensions the blood of different animals (dog [3] and horse [4]) was found to differ by the workers at Copenhagen, and not only so but it differed very materially from the dissociation curve of hæmoglobin.

(5) To explain the latter difference Bohr [3] suggested that two possible causes, (*a*) the alkalinity of the blood, (*b*) the concentration of the hæmoglobin, might affect the affinity of the hæmoglobin for oxygen, the latter increasing, the former decreasing it.

No experimental details are given to support these suggestions, nor are the differences between the curves obtained from different animals explained.

II. *Hæmoglobin.*

In our earliest experiments we found, as others had done [4], that there was a tendency for methæmoglobin to be formed when a solution of hæmoglobin was shaken at 38 °/₀ for some time. This is especially so in the presence of mercury; to avoid it we observed two precautions, (1) we were careful to use no mercury, (2) a trace of ammonium carbonate (1 drop of approximately sat. solution) was added to 3 c.c. of hæmoglobin solution. Working thus and using solutions of hæmoglobin prepared by Hoppe Seyler's method we obtained results as discordant as those of our predecessors and indeed we succeeded in imitating (though not at will) both the older and the more recent curves of Hüfner, as well as getting several intermediate curves. It seemed probable that the discordance in our results was due to the traces of ammonium carbonate added, and we consider that the starting point of our research, in so far as positive results are concerned, took place when we made a solution of dog's hæmoglobin after Bohr's receipt, dissolved the crystals in distilled water, rid the solution so obtained

of ether and determined a series of points as follows from three different solutions:

TABLE I.

Dissociation of hæmoglobin crystals dissolved in distilled water.

Solution I:					
Tension of oxygen (in mm.)	12·5	15·5	31	45	72
Percentage saturation ...	29	40	60	77·5	90·5

Solution:	II	II	II	III
Tension of oxygen (in mm.)	20	26	31·5	39
Percentage saturation ...	45	58	66	68

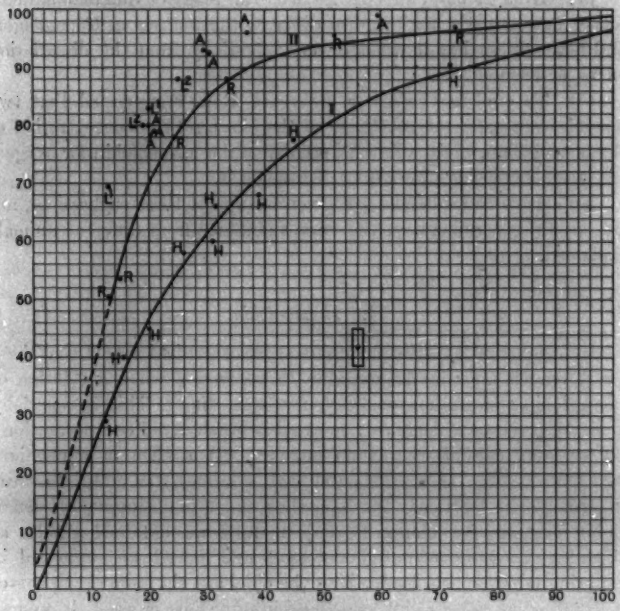

Fig. 1.

I. Bohr's dissociation curve of oxyhæmoglobin dissolved in water.

II. Dissociation curve of hæmoglobin in Ringer's solution. L^1 and L^2 represent different samples of laked blood (ammoniacal). A hæmoglobin solution made alkaline with ammonium carbonate. The rectangle around the point (at 42 % and 56 mm.) indicates the experimental error to which the determinations are liable. Temperature 37—38° C.

Fig. 1 shows the relation of the results tabulated above to Bohr's curve, they fall so nearly upon it that there can be no doubt as to the accuracy of the curve for a solution of hæmoglobin crystals in distilled water.

Such a solution cannot be regarded as chemically pure, it contains small quantities of salt which could only be removed by dialysis, and even when free from ether it has a characteristic odour similar to that of dog's blood.

A comparison of these results with those which we had previously obtained from solutions of hæmoglobin made alkaline with ammonium carbonate as already mentioned, or with blood laked by the addition of dilute ammonia, showed that there was a fundamental difference between the dissociation curves of the hæmoglobin under the different conditions of solution (see Fig. 1).

The following were the data obtained:

TABLE II.

Dissociation of hæmoglobin made alkaline by the addition of 1 *drop of ammonium carbonate to* 3 *c.c. of solution.* (*Points A in Fig.* 1.)

Tension of oxygen (in mm.)	20·5	21	20	30·5	29·5	37	59·5
Percentage saturation ...	78·5	79	80	92·5	93	96	99

TABLE III.

Dissociation of blood laked by the addition of 1·5 *c.c. of very dilute ammonia to* 1 *c.c. of blood. This ammonia solution was made by the addition of* 2 *c.c. of ammonia of sp. gr.* ·880 *to* 500 *c.c. of distilled water.*

Tension of oxygen (in mm.)	13	19	20	25
Percentage saturation ...	69	80	83·5	88

The divergence between the properties of hæmoglobin in alkaline solution and in distilled water made us anxious to study the properties of the pigment when dissolved in some medium which approximated more closely to a physiological fluid. A solution of hæmoglobin crystals in Ringer's fluid (the analysis of which is given in the Appendix) was made and its properties determined as investigated.

TABLE IV.

Successive points on the dissociation curve of Hb. in Ringer's fluid. The points as actually determined are marked R, Fig. 1.

Abscissa	10	15	20	25	30	40	50	100
Ordinate	36	56	70	79	85	91	94	99

The preceding experiment made it clear that the dissociation curve of hæmoglobin in Ringer's solution occupied a place intermediate between that of an aqueous and of a more strongly alkaline solution of the pigment.

It was not evident, however, whether the altered affinity of the hæmoglobin for oxygen was due to the slight alkalinity of the Ringer's solution, or whether it was a function of the combined effects of all the saline constituents of the fluid. We therefore crystallised some dog's hæmoglobin afresh and divided the crop of crystals into two portions, the first of which we dissolved in ·7 % NaCl and the second in distilled water. With each solution we performed experiments for the purpose of determining the readiness with which the hæmoglobin parted from its oxygen. The results are plotted in Fig. 2, points N^1—N^5.

The aqueous solution of the hæmoglobin was then investigated as a control; the points which were obtained fell on Bohr's line within the errors of experiment, they are shown in Fig. 2 as W^1, W^2, W^3.

From these data, together with a few similar observations subsequently to be described, we can set down the constants of the dissociation curve of hæmoglobin in ·7 % NaCl as follows:

TABLE V.

Ordinates (saturation) and abscissæ (tension) of points on the dissociation curve of oxyhæmoglobin in ·7 % NaCl solution.

Abscissa	10	15	20	25	30	35	40	50	60	100
Ordinate	27·5	41	60	69·5	75	79·5	83	85·5	91	98·5

This curve lies in a position intermediate between that of an aqueous solution of hæmoglobin and of a solution of hæmoglobin in Ringer's fluid.

It had now become evident that the abstract conception of a single dissociation curve of hæmoglobin as such must be abandoned, and that the curve depended not merely upon the reaction but also upon the nature and strength of the saline constituents of the solvent. The comparison of some other base with sodium suggested itself, the most natural for the purpose was potassium.

A fresh stock of hæmoglobin crystals was obtained from dog's blood by the use of Bohr's method. It was divided into three portions. The first was dissolved in ·9 % potassium chloride (equimolecular with ·7 % NaCl); the second and third portions were used as controls and were dissolved in ·7 % NaCl and in distilled water respectively.

The data obtained from the potassium chloride solution are plotted in Fig. 2, points K^{I}—K^{IV}.

Of two determinations with the ·7 % NaCl solution one was at a tension so low that the difference between the NaCl curve and the water curve is not to be distinguished (Fig. 2, N^{VI}), the difference between this point and K^{I} is however very clear. The other N^{VI} takes its place on the sodium chloride curve whilst the aqueous solution yielded a point (W^{VII}, Fig. 2) which takes its place close to Bohr's line and is clearly differentiated in saturation from N^{4} and K^{III}, though within five milli-

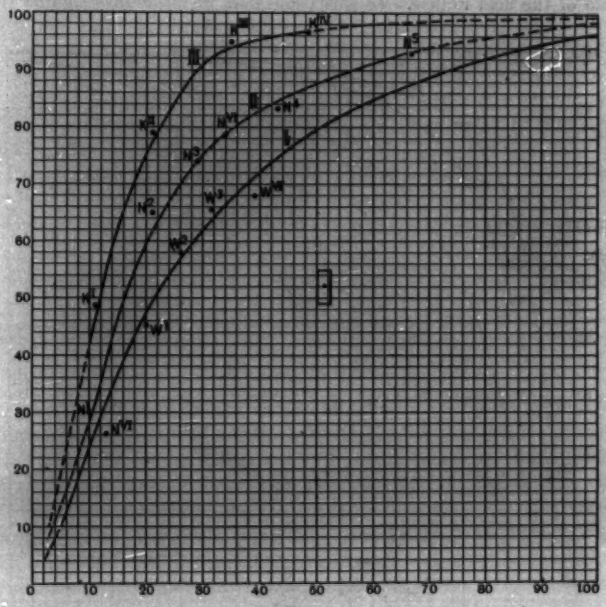

Fig. 2.

Ordinate = percentage saturation of hæmoglobin. Abscissa = tension of oxygen in mm. of mercury.

I. Dissociation curve of hæmoglobin dissolved in water.
II. ,, ,, ,, ,, ·7 % NaCl.
III. ,, ,, ,, ,, ·9 % KCl.

Rectangle surrounding point = magnitude of experimental error. Temperature 37—38° C.

metres of the same tension. The following are the constants for the dissociation curve of oxyhæmoglobin in a ·9 % solution of potassium chloride.

TABLE VI.

Ordinates and abscissæ of points on the dissociation curve of oxyhæmoglobin in a ·9 % KCl solution.

Abscissa	10	15	20	25	30	35	50	100
Ordinate	42·5	63	75·5	84·5	91	94	96	99

The dissociation curve is a function not only of the base however but also of the acid radicle. In this connexion we have investigated the

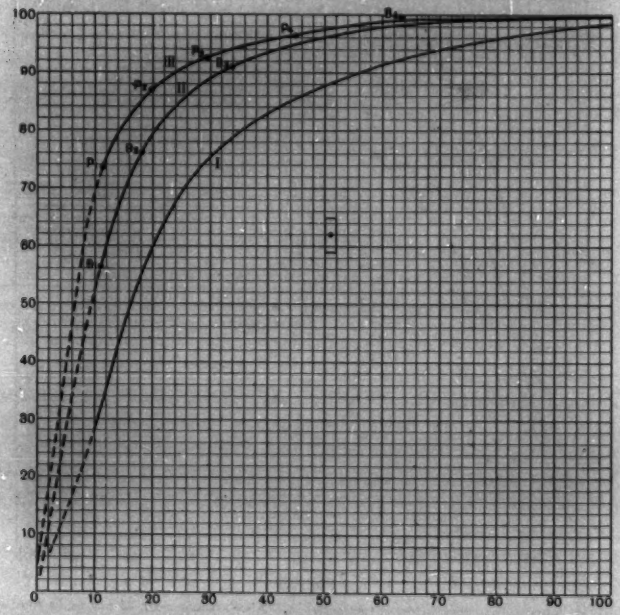

Fig. 5.

Ordinate = percentage saturation of hæmoglobin. Abscissa = tension of O_2 in mm. of mercury. Dissociation curves of hæmoglobin dissolved in

I. ·7 % NaCl, II. $NaHCO_3$, III. Na_2HPO_4,

of strengths equivalent for sodium. Temperature 37—38° C.

properties of solutions of hæmoglobin, not only in sodium chloride, but in sodium bicarbonate and in monacid sodium phosphate.

The sodium bicarbonate was of a strength equivalent in the matter of sodium to ·7 % NaCl, not therefore equimolecular, the same was the case with the Na_2HPO_4.

The data for Na_2HPO_4 are shown in Fig. 3 as points B_1—B_4.

The constants of the curve to which these data approximate are as follows:

TABLE VII.

Ordinates and abscissæ of points on the dissociation curve of a solution of hæmoglobin in 1·01 % *$NaHCO_3$.*

Abscissa	10	15	20	25	30	35	40	50	100
Ordinate	52	69·5	79	85	89	91·5	93·5	96	99·5

The corresponding data for Na_2HPO_4 are shown as points P_1—P_4 in Fig. 3.

TABLE VIII.

Ordinates and abscissæ of points on the dissociation curve of a solution of hæmoglobin in 1·6 % *of Na_2HPO_4.*

Abscissa	10	15	20	25	30	40	100
Ordinate	67·5	81	87	90·5	93	95	99·5

III. *Blood.*

Early in our experiments we were struck with the difference between human blood and that of certain animals, the cat and dog for instance. This difference had been observed by Loewy who compared his results obtained from human blood with those obtained, in conjunction with Zuntz, on dog's blood. In the actual determinations of individual samples which they investigated there was so much overlapping that the difference which appeared on the average was not very convincing, especially as the necessity for working at a specified carbonic acid tension was not then appreciated.

The effect of carbonic acid on the dissociation curve. To clear the ground we set ourselves to verify Bohr's[20] investigation of the effect of carbonic on the dissociation curve of hæmoglobin. This we did, in so far as the general principle was concerned, in the most unequivocal way. In certain questions of detail our results differ from those of

Bohr, these differences perplexed us somewhat at the time and we established them by renewed investigation. In the light of our subsequent work it is clear that they are to be explained, to some extent at all events, by the fact that we worked on sheep's blood, whilst Bohr's curves apply to the blood of the dog—to this extent our results are new and we therefore append them. They possess another slight interest inasmuch as we endeavoured to choose specified tensions of carbonic acid in studying the oxygen dissociation curve of hæmoglobin. This is a laborious process. The amount of carbonic acid which it is necessary to introduce into one of our tubes in order to give a

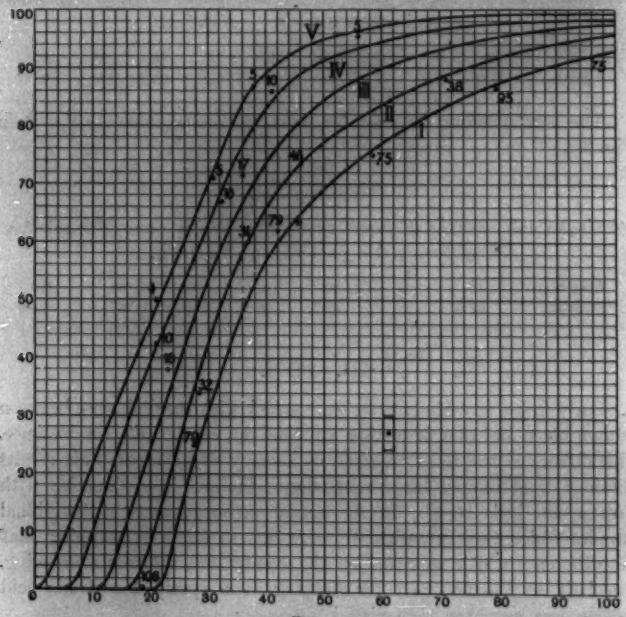

Fig. 4.

Ordinates represent percentage saturation of hæmoglobin, abscissæ tension of O_2 in mm. of mercury. Dissociation curves for sheep's blood at I 5, II 10, III 20, IV 40, and V 80 mm. tensions of CO_2 respectively. The rectangle surrounding the point shows the magnitude of the experimental error to which the observations are liable. The numbers attached to the points show the actual CO_2 tensions at which the observations were made. Temperature 37—38° C.

specified tension is, at the outset, a matter of guess work, since it depends upon unknown factors in the constitution of the blood. At the best one can only expect to be partially successful.

The results which we have obtained are plotted in Fig. 4.

The points marked on the figure as dots represent the actual determinations, whilst the figures attached to them indicate the observed CO_2 tension.

TABLE IX.

Abscissæ and ordinates of points on dissociation curves of sheep's blood at varying CO_2 tensions.

Abscissa (in mm.)	10	15	20	25	30	35	40	45	50	60	70	80	100
Ordinate:													
5 mm. Tension CO_2	28	35	47	58·5	70	81	89	93	95	96	98	99	99·5
10 mm. CO_2	11	26	38·5	51	63	74·5	83	88	91·5	94·5	96·5	97·5	98·5
20 mm. CO_2	0	10	25	49	53·5	65·5	74·5	80·5	84·5	90	93	95	97·5
40 mm. CO_2	0	0	11	26	42·5	56	65	72	77	83·5	88·5	93	95·5
80 mm. CO_2	0	0	1	12·5	31	45·5	56·5	64	69·5	77	83	87·5	92·5

As it seemed uncertain how the carbonic acid acted upon the blood we centrifugalised some dog's blood, the serum was withdrawn, the corpuscles were washed twice in Ringer's solution and divided into two portions. In the first portion the corpuscles were suspended in Ringer's solution in about the concentration of the original blood, whilst hæmoglobin crystals were made of the second and these were dissolved in Ringer's solution. In both cases it was found that CO_2 was inimical to combination with oxygen.

TABLE X.

Effect of CO_2 on corpuscles and hæmoglobin respectively.

	Washed corpuscles.			
Tension of CO_2 (in mm.)	2	5	76	89
Tension of O_2 (in mm.)	18	19	17	18
Percentage saturation	55	57	6	9

	Hæmoglobin in Ringer's solution.							
Tension of CO_2 (in mm.)	0	0	0	0	59	128	117	93
Tension of oxygen (in mm.)	18·5	33	55	78·5	18·5	33	55	78·5
Percentage saturation	66	87	94·5	98	46·5	64	87	91·5

The carbonic acid then tends to turn the oxygen from hæmoglobin, whether the hæmoglobin be contained in the corpuscle or be freely in solution.

In order to study the relation of the dissociation curves of hæmoglobin and of blood it is necessary that each should be investigated at the same tension of carbonic acid.

In the case of hæmoglobin it is simplest to work in the absence of CO_2 or at a negligible tension of say $\frac{1}{2}$—1 mm. This is not easy in the case of blood, and further the lower the CO_2 tension the greater is the effect on the curve of small variations in CO_2. Thus it is apparent from Fig. 4 that there is nearly as much difference between the dissociation curves at 5 and 10 mm. of CO_2 respectively as between 40 and 80 mm. In the case of blood, therefore, it is of advantage to work at a considerable CO_2 tension. In the following comparisons we have endeavoured to keep near to 40 mm. tension of CO_2. This quantity has a certain special interest inasmuch as it is approximately that of the blood in the body.

IV. *Relation of dissociation curves of hæmoglobin and of blood.*

It is not unnatural to suppose that the very existence of corpuscles as such may have some effect upon the dissociation curve of blood. Gases are prone to be liberated from their solutions by minute particles, and *à priori* it is not unlikely that the fact of the hæmoglobin being contained inside a small envelope of great curvature might introduce surface effects which would modify the relations of the gas within and without the corpuscle. We made it our business to ascertain whether any such physical circumstances were responsible for the differences which exist between the dissociation curves of blood and of hæmoglobin.

For any two solutions of hæmoglobin to be directly comparable it is necessary, as we have shown above, to have the hæmoglobin dissolved in the same medium as regards salts, and under the same tension of carbonic acid.

With regard to the salts our task has been rendered easy by the fact that the most excellent analyses of the salts of red corpuscles of many animals have been made within recent years by Abderhalden[8], and these with Schmidt's[9] classical analysis of human corpuscles furnish a very substantial basis on which to work.

The salts in the red corpuscles of different animals differ very much, especially as regards potassium and sodium, the corpuscles of man, pig and rabbit contain much potassium and little sodium, those of the dog and cat contain chiefly sodium, whilst the sheep occupies an intermediate position. Similar differences exist in the case of the acid radicles, in

some corpuscles the phosphates preponderate over the chlorides to a greater degree than in others. In all the analyses the bases appear to preponderate over the acid radicles, the deficiency of the latter being made up by the formation of carbonates.

Our procedure was as follows. Two animal types, man and the dog, were selected, which differed widely from one another in bases contained in their red blood corpuscles. Two portions of the same solution of hæmoglobin in distilled water were taken. To one the salts of the human red blood corpuscle (Schmidt's analysis) were added, to the other those of the dog's red blood corpuscle (Abderhalden's analysis). The way in which the solution was made up was as follows. A graduated measuring cylinder of 25 c.c. was taken; into it was put as much normal HCl as was necessary to provide the calculated quantity of chlorine for 20 c.c. of fluid. This in the case of man is 1 c.c. precisely. To this is added the weighed quantity of phosphoric acid. The analyses are given as P_2O_5, we used metaphosphoric acid in anhydrous sticks, weighing out the quantity which would provide the calculated amount of P_2O_5. To the acid solution in the bottom of the cylinder we added the K and Na in the form of bicarbonate, the excess of CO_2 was shaken off and we were left with about 1 c.c. of fluid containing the required amount of K, Na, Cl, P_2O_5 and HCO_3. Calcium, magnesium and sulphate figure to so small a degree in red blood corpuscles that we determined, in the first instance at all events, to neglect them. Hæmoglobin solution (13 %) in water was then poured into the cylinder till the whole was made up to 20 c.c. In the case of each solution (that which simulated human corpuscles and that which simulated those of the dog) several points on the dissociation curve were determined. The data obtained were as follows:

TABLE XI.

Dissociation of a solution of hæmoglobin in a solution of the salts (K, Na, P_2O_5, Cl and HCO_3). Of the concentration in which they exist in the human corpuscle. (Points H_1—H_4 in Fig. 5.)

Tension of CO_2 (in mm.)	34	41	37	40
Tension of O_2 (in mm.)	16	20	40	73
Percentage saturation	33	44	78·5	94

TABLE XII.

Dissociation of the same solution of hæmoglobin made up with the salts of the dog's red blood corpuscle. (Points H^1—H^4 in Fig. 6.)

Tension of CO_2 (in mm.)	42	43	42	49
Tension of O_2 (in mm.)	21	32	52	68·5
Percentage saturation	23	35·5	73	87·5

For direct comparison with these the dissociation curve of human blood and of dog's blood under similar conditions was investigated with the following results:

TABLE XIII.

Dissociation curve of human blood (defibrinated by whipping) at about 40 mm. Tension CO_2. (Points B_1—B_{12} in Fig. 5.)

Tension of CO_2 (in mm.)	40	45	44	38	38	29·5	37	43·5	45	40·5	31·5	35·5
Tension of oxygen (in mm.)	5·5	8	13·5	16	27	28·5	41·5	45·5	47	57·5	87	159
Percentage saturation	1·5	1·5	9·5	33·5	66·5	62	82	85	88	88	92	97

TABLE XIV.

Dissociation curve of dog's blood (defibrinated by whipping). (Points B^1—B^7 in Fig. 6.)

Tension of CO_2 (in mm.)	37·5	42	40	37	47·5	43·5	39
Tension of oxygen (in mm.)	19	36	41	47·5	58	84	105
Percentage saturation	19·5	43	53	65	76·5	88	91

The direct comparison of the blood, whether human or canine, with the corresponding hæmoglobin solution, as exhibited in Figs. 5 and 6, reveals the fact that the points proper to each animal fall on a single curve which is characteristic of that animal. A hæmoglobin solution made up with the salts of the human red blood corpuscles has a dissociation curve indistinguishable from that of human blood, whilst the same hæmoglobin solution to which are added the salts of dog's red blood corpuscles has the same dissociative properties as dog's blood.

The corpuscle then profoundly influences the relation of the oxygen to the hæmoglobin within it, not by any surface effect, but in virtue of its being a receptacle of certain inorganic salts which are different from those of the medium which surrounds it.

Since the curve given in Fig. 5 represents, as nearly as our present knowledge will permit, the dissociation curve of blood as it circulates in the human body we have determined no less than twelve points on the curve. Previous to some of the determinations we pumped the oxygen from the blood and started our observation on reduced and not on saturated blood. Indeed it is only possible to obtain determinations at points of such low tension as B_1 and B_2 by the addition of this procedure.

The following table shows the relation between the ordinate and

abscissa of various points in the dissociation curves of human and of dog's blood respectively:

TABLE XV.

Blood at approximately 40 *mm. tension of* CO_2 *and* 37—39° *C.*

Abscissa	10	15	20	25	30	35	40	45	50	60	80	100	160
Ordinate for human blood	0	21·5	45·5	59·5	69	75·5	80	83·5	86	88·5	91·5	93·5	97
Ordinate for dog's blood	5	11·5	18·5	20·5	32·5	41·5	50·5	60·5	69	80	88·5	90	—

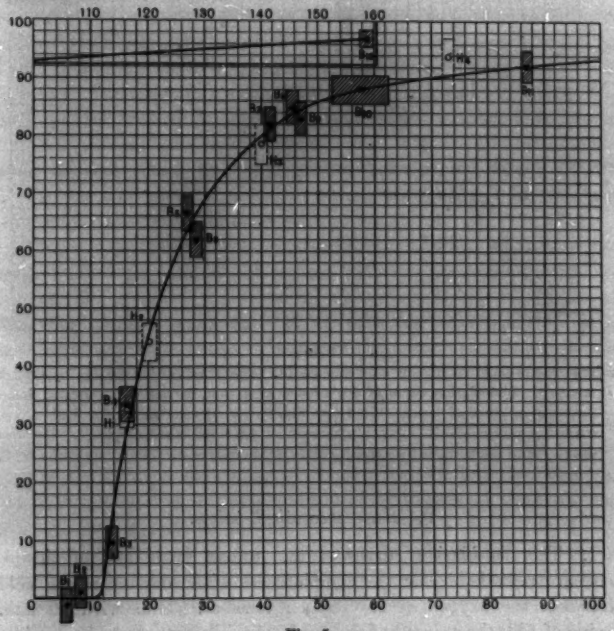

Fig. 5.

Ordinate = percentage saturation. Abscissa = tension of O_2 in mm. of mercury. Dissociation curve of human blood at 40 mm. tension of CO_2. • = determination for human blood. o = ditto for haemoglobin solution with salts of human red blood corpuscles. The area drawn round the point is the experimental error in each case. Temperature 37—38° C.

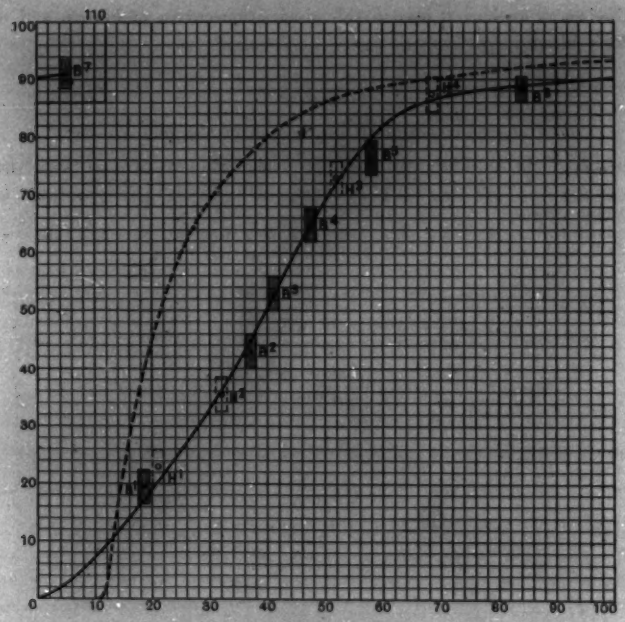

Fig. 6.

Ordinate = percentage saturation of hæmoglobin. Abscissa = tension of O_2 in mm. of mercury. Dissociation curve of dog's blood at 40 mm. tension CO_2. Dotted line = dissociation curve of human blood (see Fig. 5). • = determination for dog's blood. ○ = ditto for hæmoglobin solution with salts of dog's red blood corpuscle. The area drawn round the point is the experimental error in each case. Temperature 37—38° C.

V. *Some theoretical points.*

1. The dissociation curves of the human blood and of sheep's blood are remarkable for the completeness with which carbonic acid eliminates the oxygen at low oxygen tensions. Thus with 40 mm. tension of CO_2 the amount of oxygen in blood is immeasurably small at oxygen tensions of 15 mm. and under. The value of blood as a medium for respiration depends upon (1) the readiness with which it will take oxygen up in the lungs and (2) the readiness with which it will dispose of it to the tissues. In both of these respects, but especially in the

latter, blood has the advantage over a hæmoglobin solution. In this connexion it is instructive to compare Fig. 5 with Fig. 1. The curve of blood as it exists in the body crosses the curve of a simple hæmoglobin solution at a tension of about 20 mm. of oxygen, above this tension blood, owing to the presence of salts in the corpuscle, chiefly of potassium salts, has a much greater attraction for oxygen than a hæmoglobin solution would have, at 30 mm. oxygen tension for instance the hæmoglobin solution is 62 % saturated, whilst the blood is 69 % saturated, on the other hand at 15 mm. the hæmoglobin solution retains 36 % of its oxygen whilst the blood is practically deoxydised. There seems then to be a very good reason why the respiratory processes of the body are so adjusted as to keep up an adequate tension of carbonic acid in the blood. The point of intersection of the two curves must depend upon the conditions prevailing both in the lungs and in the tissues. At great heights, for instance, the taking up of oxygen by the lungs becomes more difficult, it is reasonable then that the blood curve should shift somewhat to the left in order that the oxygen may be taken up more readily by the lungs, and that a portion of this burden of disadvantage should be thrown upon the tissues, which means ultimately that their activity would be restricted.

That this is what actually occurs at high altitudes has been shown by Ward[9] whose alveolar CO_2 tension fell from 37·7 mm. in London to 28·5 on Monte Rosa. The tuning of the respiratory centre to an abnormally low CO_2 tension at high altitudes aids pulmonary respiration in at least two ways, firstly it increases the amount of oxygen in the alveolar air and secondly it increases the affinity of the alveolar blood for oxygen.

2. The limiting pressure of oxygen in the alveolar air is stated to be 30 mm. approximately. This fact presents itself with much more force in the light of the curves which have been determined by Bohr and by ourselves than in those determined by Hüfner. The tension of 30 mm. means but 69 % saturation in human blood. Now the numerous observations of Hill and Nabarro[10] indicate that in the dog muscle depletes the blood of 70—80 % of its normal quotum of oxygen. Zuntz[11] found an even greater absorption. If this figure is true for man it is clear that blood containing less than 70 % of its usual quantity of oxygen and circulating at the usual rate will fail to satisfy the muscle. Therefore if the muscle is not to suffer from deficiency of oxygen the circulation must quicken and the muscle must either receive blood at the expense of other organs or extra work

must fall upon the heart. We may push our enquiry somewhat further and say a word about each of these alternatives. The heart, itself a muscle, is at the same initial disadvantage as the rest of the musculature of the body and therefore can only ward off its own asphyxia at even its normal rate of working by increasing its blood supply, to require increased work of it is therefore to set up a vicious circle. As regards other organs we need only mention two, the kidney and the brain. These are probably even more sensitive to want of oxygen than muscle. Normally the blood leaving the kidney [12] contains more than 60 % of its oxygen. The following figures show that the blood usually emerges from the kidney containing oxygen at a higher tension, and in three out of four cases cited at a much higher tension than that of the oxygen in the arterial blood which would enter the kidney under conditions which would allow the blood to be only 69 % saturated with oxygen (assuming that the quantities of oxygen in the blood involved are the same for man as for the dog).

Oxygen in arterial blood in c.c. per 100 of blood	Oxygen in venous blood in c.c. per 100 of blood	Percentage sat. of venous blood (art. blood taken to 97 % saturated)	Tension of oxygen in venous blood (human curve) in mm.
23·2	21·7	91	77
13·4	9·7	70	31
17·8	16·9	92	80
24·2	23·6	94	119

As regards the central nervous system we have less information, since the only analyses which exist are of blood from the Torcula and which therefore comes from the meninges and other places as well as the substance of the brain itself. The data of Hill and Nabarro [13] show however that this blood, like that of the kidney, is very arterial. If the blood went to the brain 69 % saturated, and the quantity of oxygen were taken out which was observed by these authors (about 20 % of the whole oxygen capacity) the blood would leave the brain 49 % saturated. The oxygen tensions of the blood entering and emerging from the brain would then be 30 and 21 mm. of mercury respectively. Normally the blood leaves the brain about 80 % saturated, corresponding to a tension of 40 mm. of mercury. Haldane and Boycott [23] have found that an oxygen tension of 60 mm. of mercury in the alveolar air affects the respiratory centre under normal conditions. Apart from the question of secretory activity of the lungs this would correspond to 88 % saturation of the arterial blood and 68 % saturation (tension 29 mm.) of the venous blood (assuming that the blood-flow does not quicken), *i.e.* the venous blood would leave the respiratory

centre at a tension which is approximately that of the oxygen in the arterial at an altitude at which life becomes impossible.

In all these cases the tension of oxygen in the venous blood might be and probably is raised by local vascular dilatation. It is hardly necessary to point out how great would be the strain on the vascular system of endeavouring to maintain the blood-pressure.

3. In what has just been said we have assumed that the arterial blood is in equilibrium with the alveolar air and therefore we have ignored for the moment the secretory theory of respiration. This theory rests chiefly on the ærotonometric work of Bohr, the theory of the invasion and evasion coefficients put forward by the same author, and the determinations of Haldane and Lorrain Smith[14] of the oxygen tension in the alveolar air by the carbon monoxide method. It is clear that the considerations put forward in this paper must affect the interpretation of Haldane and Lorrain Smith's figures to a greater or less extent. Probably it would be more satisfactory to redetermine the relative saturations of blood with oxygen and CO in the presence of CO_2 than to attempt to recalculate their results from the data at present at our disposal.

4. Whilst our experimental determination of the dissociation curve of hæmoglobin agrees precisely with that of Bohr, this very agreement forms a criticism of Bohr's[13] theoretical handling of his data, for he, in opposition to Hüfner, maintains that the concentration of the solution should influence the form of the curve, it should be pointed out very clearly therefore that whilst Bohr's curve was obtained with a six per cent. solution of hæmoglobin our curves were obtained with twelve to fifteen per cent. solutions as determined from our oxygen readings and checked in most cases by a standard Haldane's hæmoglobinometer.

The practical advantage of solutions of this strength, which may be obtained by shaking the crystals with water a little at a time at 38° for about three hours, are very great, inasmuch as the correction necessary for the oxygen physically dissolved in the fluid is correspondingly small.

5. The formula published recently by Wolfgang Ostwald as representing the dissociation curve of hæmoglobin, $\frac{x}{a} = k.c^m$, when x = the quantity of gas absorbed, or in his phraseology "adsorbed," a the quantity of blood (or presumably of hæmoglobin), c the pressure of gas, and k and m constants, is one in which the concentration of the hæmoglobin does not influence the actual form of the curve and is to this extent consistent with our experimental results.

Appendix.

The methods employed.

The determinations just discussed have been obtained by a method which is much more rapid than those of previous workers and it involves the use of only small quantities of blood or hæmoglobin solution. Therefore it has become possible to undertake investigations of the dissociation of hæmoglobin under various circumstances on a scale which was quite impracticable with the ærotonometer and the blood gas-pump.

The ærotonometer in the present case is reduced to a simple glass tube of about the size of a test-tube.

This tube was of the pattern described by Bohr for the purpose of collecting gases from the blood gas-pump. It consists of a glass tube open at one end, of length 18 cm. and diameter about 1·3 cm.; at the other end is a three-way "tail-tap," the bore of this and of the tubing immediately connected with it is about 2mm.

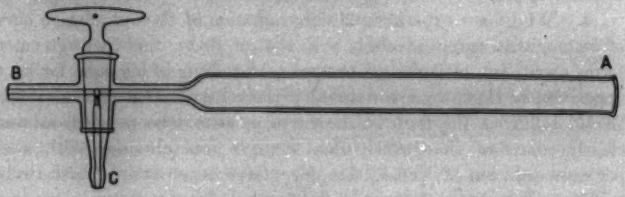

Fig. 7.

Into this is placed $2\frac{1}{2}$ c.c. of hæmoglobin solution, it is then inverted so that it stands vertically, the end *A* being immersed in mercury. The air is sucked out from the end *B* and a piece of rubber tubing which leads from the nitrogen bottle is attached to *B*. The tube contains now only mercury and hæmoglobin solution, a known quantity of air may be introduced at the end *A* over mercury from a measure which consists of a test-tube of the required size. Nitrogen is then introduced at *B*. The tap of the nitrogen cylinder is opened. The gas is allowed to go to waste through *C* until all air which may have been in the connexions is swept out. The tap is then cautiously turned so that the nitrogen goes into the ærotonometer tube. It is allowed to fill the tube until the mercury-hæmoglobin surface has sunk to within a few

millimetres of the end *A* of the tube. The tube is then corked with a rubber cork and inverted so that *B* is downwards. Any mercury which is in the tube is allowed to run out. It is well that the cork should be driven a good way into the tube; when the mercury has run out the pressure inside the tube will still be slightly in excess of that of the atmosphere. The tap is therefore given a rapid turn (with the tube horizontal) so that the two pressures may be equal. The experiment is therefore commenced with the gas at the atmospheric pressure. The ærotonometer tube is placed in the bath at 37—38° and shaken as before. At the end the pressure will have changed for several reasons, (1) the gas is heated, (2) there will be increased tension of aqueous vapour, (3) there will be an exchange of gas between the hæmoglobin and the blood. The volume of the gas is of course constant throughout. These causes of variation in the pressure are of course all calculable quantities, and the third has been left out of account however except in cases where the exchange produced an appreciable alteration of pressure—for instance where a known but considerable quantity of carbonic acid was introduced and subsequent gas analysis showed that the blood or hæmoglobin solution absorbed quantities of the order of 1 c.c. which made a difference of about 5 % to the pressure. In such cases this factor was taken into account.

In the ordinary determinations the following example will show that the inclusion of this factor is unnecessary. Consider a 10 % hæmoglobin solution at approximately 10 mm. pressure of oxygen. If it were saturated when introduced in the apparatus it would be about half saturated at the end, that is to say 2·5 c.c. of it would yield up approximately ·2 c.c. of oxygen. The volume of the tube is about 20 c.c., therefore there would be an error of 1 % in the total pressure of gas in the tube at the end of the experiment. Now the oxygen tension is approximately 10 mm. and is in error 1 % and therefore ·1 mm. But the method professes at most to be exact to 1 mm.

Again suppose the oxygen pressure to be 50 mm. the saturation being about 80 %, 2½ c.c. of blood will give up ·8 c.c. The error caused by this will be ·4 % or ·2 mm. in 50 mm.

When the tube had been sufficiently shaken in the bath it was removed, held vertically with the cork downwards, gripped for a moment with a cold wet towel, care being taken not to shake the tube, the cork was then removed from the tube under mercury and as quickly as possible the necessary fluid for analysis was taken out with a special pipette.

This was of the form shown in Fig. 8. The curved portion, *A*, was of tubing of about one millimetre bore as also was the portion *C*. The length of *C* must be sufficient to allow the end to reach the bottom of one of the blood gas bottles of the differential blood gas apparatus. The major portion of the pipette is one cubic centimetre in capacity between two marks and is made of tubing of about 2 mm. bore. The curved end is immersed in the mercury and slipped into the eudiometer tube. Care must be taken to see that the curved portion fills itself with mercury, otherwise a direct air passage is formed into the eudiometer and air may be sucked in.

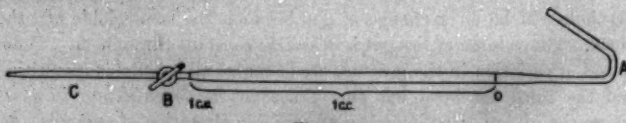

Fig. 8.

Blood is withdrawn from the eudiometer till the blood surface reaches the tap *B*. The tube is then removed and inverted. Any mercury which is in it falls to the region of the tap. When the tap is opened therefore a globule of mercury first flows down the portion *C*. This is followed by blood. Fluid is allowed to run out until the upper meniscus reaches the zero mark. The tube is now practically a burette. The extremity of which is placed in the blood gas bottle. It should touch the bottom of the bottle. In the bottle is $1\frac{1}{2}$ c.c. of ammonia. The blood is therefore delivered gently and lies in a layer underneath the ammonia solution.

When the hæmoglobin solution or blood, as the case may be, has been taken out of the tube the upper end *B* (Fig. 7) may be connected to a Haldane's gas analysis apparatus and a sample of the gas withdrawn and analysed, usually the analysis of the gas was postponed till after the analyses of the fluid had all been made. Under such circumstances the corks were replaced in the tubes and the tubes put back in the warm bath.

The analysis of the gases has been performed in that form of apparatus which was described by Haldane for the analysis of air. The gas burette was of 10 c.c. capacity, the bulb 7 c.c. in size and the stem was divided in hundredths of a c.c. The samples of gas used by us for analysis were usually of about 8 c.c. Taking this quantity a hundredth of a c.c. of oxygen corresponded to a tension of about 1 mm.

of mercury. As there was no difficulty in reading the meniscus to half a division, *i.e.* to 1/200 c.c., the apparatus was sufficiently accurate for our purpose.

In our earliest observations we made duplicate analysis of the gas in the tube. This however we found later to be an unnecessary complication.

The analysis of the blood gases. The blood has been analysed in the differential method. The apparatus used is in all essentials that described by one of us in this *Journal*, but one or two alterations in detail have been introduced.

(1) Egg-shaped bottles have been used by us in some of our apparatus. This provides for the ammonia lying in a thicker layer over the blood than was the case with the cylindrical or conical bottles.

(2) The arrangement for raising and lowering the fluid in the manometers has been modified.

The percentage saturation of oxygen is found in the following way. Suppose A and B to be the two bottles of the apparatus. 1·5 c.c. of NH_3 is put in each and in A is put 1 c.c. of the blood for analysis, in B 1 c.c. of the same blood which however has been saturated with oxygen. The difference between the oxygen in the two samples of blood is then determined. Let this be $d' \times c$, where d' is the observed difference of pressure and c the constant of the apparatus. When the measurement has been made it is necessary to measure the total quantity of oxygen which A now contains. This is done with ferricyanide in the manner described in a former paper. Let this be $D \times c$. The percentage saturation would then be $\frac{d' \times c}{D \times c} \times 100$, but for a slight correction which must be made for the oxygen dissolved in the plasma. $d' \times c$ is the difference between the amount of oxygen taken up in bottle A and in bottle B—the latter is of course zero since the blood in bottle B was saturated previously to the determination—$d' \times c$ therefore becomes the amount of oxygen taken up by blood A. Now this is taken up by both the plasma and the corpuscles. The simplest way of eliminating the quantity of oxygen taken up by the plasma is to find out what pressure in the manometer it represents at different tensions of oxygen and subtract this from the total reading. In the apparatus we have used it corresponds to 2 mm. at a tension of 0 mm. of mercury, 1·5 at 38 mm., 1 at 76 mm., above which tension we have worked but seldom, let this difference of pressure be p then, d the difference of pressure due to the oxygen absorbed by the hæmoglobin of the blood in A is equal to $(d' - p)$.

Since the ferricyanide turns the oxygen from the hæmoglobin only there is no such correction in the division of the fraction, and therefore the corrected percentage saturation

$$S = \frac{100 \times d \times c}{D \times c} = \frac{100\,(d'-p)\,c}{D} = \frac{100\,(d'-p)}{D}.$$

The fact that the constant of the apparatus does not come into the calculations renders the calculations much simpler.

It is important to observe that the ratio of p to D is small. The blood of different animals differs considerably in the quantity of hæmoglobin present. Usually however D varies from about 45 mm. in the cat to about 70 mm. in the dog, p therefore varies from about 4 to about 2 % of D.

In the case of hæmoglobin solutions it might easily be otherwise. Most previous workers have made their observations on hæmoglobin solutions of a concentration of about 5 %. For such a solution D would be about 20 mm. and therefore p would be as much as 10 % of D. It will be obvious therefore that p may become a very considerable quantity relatively to d', for it is only at very low tensions that d' is more than half D. We have therefore endeavoured to obtain as strong hæmoglobin solutions as possible, and for the most part we have been successful in getting solutions which contain as much hæmoglobin as does blood and which frequently gave readings of 110—120 on Haldane's hæmoglobinometer. We obtained solutions by the very gradual addition of water to the crystals which separate from the etherial solution combined with incessant shaking at 38° C.

Analysis of CO_2 used in experiments.

$CO_2 = 98{\cdot}93$ %, $N_2 = 0{\cdot}82$ %, $O_2 = 0{\cdot}25$ %, $CO = 0{\cdot}000$ %.

Analysis of Ringer's Solution used in experiments.

NaCl 6·3 gr., KCl 0·25 gr., $CaCl_2$ 0·45 gr., $NaHCO_3$ 0·15 gr., distilled water one litre.

Accuracy of analyses. The calculated experimental error is shown as a rectangle in each of the Figures 1—6. The meaning of the rectangle is this, that if the point determined (say in Fig. 3) is the point in the middle of the rectangle the dissociation curve would fall within the specified area, there being the possibility that the saturation as measured was wrong to the extent of 2 % which might be in either direction, whilst the tension should not be more than 1 mm. wrong—*i.e.*

the oxygen reading should not be out more than ·01 c.c. in about 8 c.c. of gas; the gas burette read easily to ·005 c.c.

That the observed errors were within the calculated limits are shown by the following observations where duplicate analyses were made.

1. Sheep's blood tension of oxygen 88 mm., duplicate samples of blood were analysed giving saturations of 82 % and 83 % respectively (uncorrected for oxygen in plasma).

2. Sheep's blood tension of oxygen 54·5, saturation 70 and 67·5 % (ditto).

3. Hæmoglobin solution (alkaline); three tubes similarly treated.

	(*a*)	(*b*)	(*c*)
Tension (in mm.)	20	20·5	21
Percentage saturation ...	80	78·5	79

4. Washed corpuscles (in presence of CO_2); two tubes similarly treated.

	(*a*)	(*b*)
Tension (in mm.)	18	19
Percentage saturation ...	55	57

5. Washed corpuscles (in absence of CO_2); two tubes similarly treated.

	(*a*)	(*b*)
Tension (in mm.)	17	18
Percentage saturation ...	6	9

6. Hæmoglobin solution (alkaline); two tubes similarly treated.

	(*a*)	(*b*)
Tension (in mm.)	29·5	30·5
Percentage saturation ...	93	92·5

Summary.

1. Curves of the dissociation of hæmoglobin in water and in certain saline solutions have been determined.

2. Confirmation has been obtained of the dissociation curves of hæmoglobin in water as observed by Bohr and of the effect of CO_2 in dissociating the oxygen from blood.

3. The dissociation curve of human blood has been determined at 38° and 40 mm. tension of CO_2.

4. This curve is contrasted with that of dog's blood under similar circumstances.

5. The differences between the dissociation curves of human and dog's blood are to be explained by the differences in the saline constituent of the red blood corpuscles of the respective animals.

6. Some theoretical application of the data given in the paper are considered.

(The cost of this research has been largely defrayed by a grant from the Royal Society.)

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THE DISSOCIATION CURVE OF HÆMOGLOBIN.

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(*From the Physiological Laboratory, Cambridge.*)

THE mathematical form of the dissociation curve of hæmoglobin and its theoretical significance are subjects which have been discussed by several observers.

Hüfner[1] started from the theoretical standpoint that the association of hæmoglobin and oxygen was to be represented as a mass action with the formula $Hb + O_2 \rightleftarrows HbO_2$. If then, in a given solution the concentration of reduced hæmoglobin be C_r and of oxyhæmoglobin C_o and that of oxygen $\frac{p_o \times \alpha_t}{760}$, where p_o is the partial pressure of oxygen and α_t the absorption coefficient of the gas at the temperature t, it would follow that if k is the velocity constant

$$k = \frac{C_o \times 760}{C_r \times p_o \times \alpha_t} \text{ or } \frac{C_o}{C_r \times p_o} = \frac{k \times \alpha_t}{760}.$$

Since the quantities on the right of the equation are constant for a given temperature they may be called κ.

The first portion of Hüfner's research depended upon the experimental determination of κ; for this purpose p_o was determined by direct observation and $\frac{C_o}{C_r}$ was determined by the spectrophotometer; and secondly as α_t could also be determined it was possible to find k and therefore to draw the rectangular hyperbola which should represent the dissociation curve on the theory of mass action.

When Bohr[2], by the actual determination of a series of points for a given solution obtained a dissociation curve which was not a rectangular hyperbola little, except some statement of its inherent probability, could be said in favour of Hüfner's work. The fundamental conception on which it was based had not been supported by

Bohr's facts and Hüfner himself had adduced none in its favour. Bohr showed that his own curve approximated very closely to what would be theoretically correct on the assumption a double dissociation took place, namely

(1) $H \rightleftarrows G + F$ (in which H is the hæmoglobin) whilst G and F are the protein and iron holding moieties respectively.

(2) $F_o \rightleftarrows F + O_2$ in which F_o and F represent the iron holding moiety united and ununited to oxygen respectively.

Wolfgang Ostwald[3] has explained the available data on the assumption that the oxygen is not chemically united with the hæmoglobin, but is absorbed.

In a paper recently written by Barcroft and Camis[4] there are several points which have some bearing on the questions treated by the authors named.

(1) They confirmed Bohr's curve in the case of more than one hæmoglobin solution.

(2) This confirmation seemed to be independent of the concentration of the solution—a point which conflicts with Bohr's theoretical deductions.

(3) They showed that the form of the curve depended on the concentration of the salts present.

It therefore seemed desirable to determine the dissociation curve of a hæmoglobin solution which was not only much more nearly salt free than those previously in use, but in which the residual concentration of salts was defined.

The simplest method of attaining the latter object was to determine the electric conductivity of the solution. In the case of the fluid used for the present research the conductivity was determined by Mr W. B. Hardy, who has been unfailing in his kindness to us, not only in this respect, but in giving us the benefit of his knowledge of the technique of purifying protein solutions.

The solution of hæmoglobin was made as follows:—Dogs' blood, freshly drawn, was whipped, centrifugalised, and the serum drawn off, Ringer's fluid was then added, to make up to the original bulk, and the corpuscles shaken up. The corpuscles were given three washings with Ringer's fluid in all and then ether added and the mass of corpuscles stirred until a crystalline paste was obtained; this was allowed to stand in the cold over night. The etherial mass was then warmed up to 40° and centrifugalised in warm tubes. The supernatant fluid and the upper portion of the crystalline layer were discarded, the lower portion was slowly dissolved, with constant shaking in distilled water at 40° until the etherial smell had disappeared and only the characteristic smell proper to dogs' blood remained. The solution so prepared was filtered through several layers of filter paper on a Nutsche filter and divided into two portions A and B.

Portion A was freed from salts as far as possible by dialysis. The essential difficulty about this operation arose from the time which it took, seeing that there must be no bacterial action in the solution. The following course was adopted. A stoppered glass cylinder of about 10 inches in height and three inches in diameter was employed for the dialysis. It was thoroughly rinsed with formalin and the formalin removed by repeated washings with boiled distilled water. The hæmoglobin solution was placed in a parchment tube of the ordinary form. This tube was suspended in boiled distilled water in the glass cylinder. The tube like the cylinder was rendered aseptic with formalin and similarly washed. The dialysis was allowed to proceed for two days during which time the cylinder stood in ice. The water was changed at intervals of a few hours during the day-time. The earlier samples of water which had been used for the dialysis gave a precipitate when tested with silver nitrate, the later ones did not. At the end the hæmoglobin solution was without odour of any kind. Clearly then the cause of its original odour, as well as all suspicion of formalin had disappeared.

During the dialysis the hæmoglobin solution became considerably diluted and at the end it gave a reading of approximately 30 with Haldane's standard hæmoglobinometer. It was necessary therefore to concentrate it. This was done by distillation over a water bath at 40° in an ordinary distillation flask fitted to a condenser. The condenser was surrounded by ice; the whole was kept vacuous by the action of a Tœpler pump fitted with a drying chamber which contained sulphuric acid. The concentration was allowed to proceed until the hæmoglobin solution gave a reading of 70 on the hæmoglobinometer—which was identical with that of solution B.

Portion B was kept in ice during the dialysis of A. To start with it had a hæmoglobin value of 85, before use it was filtered through a new Berkefeld filter which reduced its hæmoglobin value to 70.

The dissociation curves of solutions A and B were determined. The methods of investigation were in all respects those described by Barcroft and Camis.

The results are given in figure 1. The encircled dots represent the points given by the dialysed solution (A) whilst the squares those given by the undialysed solution (B).

Two curves are shown on the figure of which I is a rectangular hyperbola the asymptotes of which correspond to 100° saturation and −8 mm. pressure. The hyperbola has the equation $xy = 800$. It therefore passes through the zero point both of tension and saturation, and approximates to the condition of complete saturation. II is Bohr's dissociation curve of hæmoglobin solution which has been verified on numerous occasions (Barcroft and Camis).

The points determined from solution B fall so nearly upon Bohr's curve as to form a further confirmation of it; but with the dialysed solution it is otherwise.

The dissociation curve approximates so closely to the theoretical limit as to make it entirely reasonable to suppose that this limit would be reached could the hæmoglobin be freed from the last traces of salt.

(The determination of the electric conductivity of the solution showed that it was equivalent to a 0·004 N. solution of sodium chloride.) The dialysed solution then conforms very closely to the theoretical formula of a simple reversible action.

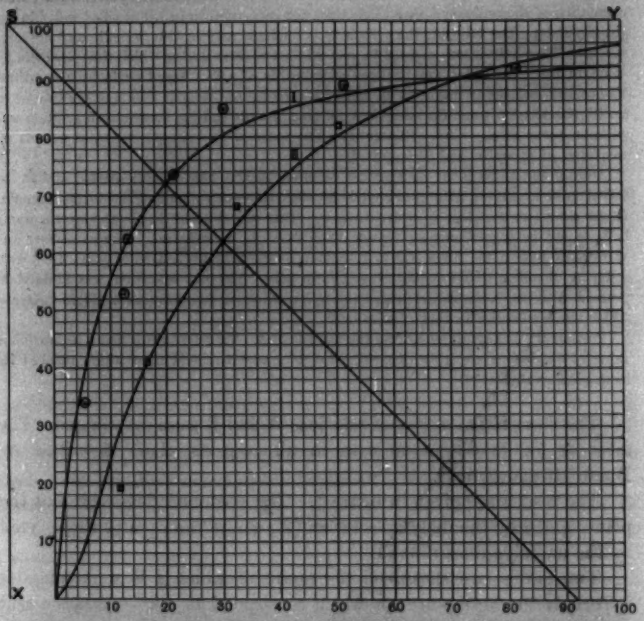

Fig. 1. Ordinates = Percentage saturation of hæmoglobin with oxygen. Abscissæ = tension of oxygen in mm. of mercury. Curve I = rectangular hyperbola. $xy = 800$. Curve II = Bohr's dissociation curve of hæmoglobin.

⊙ Points determined from dialysed solution.

□ Points determined from undialysed solution.

Having so far confirmed the hyperbolic form of Hüfner's curve it remained to compare the actual curves obtained, *i.e.* in effect to consider the divergence of the values of k in Hüfner's curve and in ours. Now the value of k in Hüfner's curve depends directly upon the value of κ and herein lies the weak point in Hüfner's determinations, for κ in the case of different hæmoglobin solutions varied from 0·07 to

0·15. The figure he finally uses, 0·11, is not even the mean of his determinations but the mean of three other means, each of which belongs to a series and these three series contain very unequal numbers of individual experiments.

Nevertheless the results obtained by Hüfner and by ourselves are extraordinarily similar and this similarity is the more satisfactory when account is taken of the wide difference between the methods employed, both of preparation of the solutions and of observation of their properties.

The divergence between the two hyperbolæ will be greatest at points where they cross their transverse axes. At 20 mm. tension of oxygen the curve in Fig. 1 shows a saturation of 72 %, whilst Hüfner obtained 69 %, indicating a difference which is not appreciable when the experimental errors of both sets of observations are taken into account.

Two points remain which demand a word of discussion:

(1) It is quite uncertain how the salts influence the form of the curve. Among the various possibilities one would be that they combine with one or other portion of the hæmoglobin and thus induce the dissociation which Bohr supposes to take place. It is possible therefore that Bohr's explanation as well as his curve is in essence correct for the hæmoglobin solutions which he used. The point which we affirm is that the dissociation of the protein from the iron holding portion of the molecule is not fundamental to the dissociation of the oxygen.

(2) With regard to W. Ostwald's contention that the oxygen is absorbed it should be said that the hyperbolic form of the dissociation curve does not necessarily preclude his hypothesis. But since the hypothesis is put forward for the purpose of explaining a dissociation curve of a different type and since the hyperbolic type is demanded by the laws of mass action there seems to be no good reason for departing from the view till recently held by physiologists: viz. that the oxygen is united chemically with the hæmoglobin and that this union is fundamentally a mass action of the type $Hb + O_2 \rightleftarrows HbO_2$.

(The cost of this research has been largely defrayed by a grant from the Royal Society.)

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THE CONDITIONS OF TISSUE RESPIRATION. Part III. THE ACTION OF POISONS.

By H. M. VERNON.

(From the Physiological Laboratory, Oxford.)

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It is self-evident that every substance which acts upon a living tissue must enter into intimate relationship with certain constituents of that tissue. Probably in the great majority of cases this relationship is a chemical one. In 1899 Loeb[1] suggested that when a living tissue is placed in a solution of an electrolyte, the ions diffuse into it, and chemically combine with some constituents, presumably proteid, to form what he termed an "ion-proteid" compound. Pauli[2] arrived independently at the same conclusion, and the hypothesis has been supported by much subsequent work[3]. Many of these ion-proteid compounds are very unstable, and are rapidly dissociated in the presence of other ions, which replace them according to their relative masses.

[1] Loeb. *Pflüger's Arch.* LXXV. p. 303. 1899. *Amer. Journ. Physiol.* III. p. 327. 1900.

[2] Pauli. *Ueber Physikalisch-Chemische Methoden und Probleme in der Medicin.* Wien. 1900.

[3] Cf. T. Brailsford Robertson. *Report of Australasian Association for Advancement of Science*, XI. p. 490. 1907.

But some of them, such as the combinations with the salts of the heavy metals, are insoluble and non-dissociable compounds. Intermediate in stability between these extremes are found compounds such as those between certain alkaloids and the tissues, which form non-diffusible complexes. They have been investigated in some detail by T. B. Robertson[1].

In investigating the action of poisons upon the phenomena of tissue respiration, I have made experiments with each of the three classes of substances mentioned: but the special object of this research was to study the action of certain members of the third class, *i.e.* of bodies which enter into definite and fairly stable combination with certain constituents of the tissues, but which can be induced to break away from such combination under suitable conditions. I hoped that by such means some information might be obtained as to the nature of the intimate chemical processes upon which tissue respiration depends.

The method of experiment is one which has already been described in previous papers[2]. The most convenient organ available, viz. the kidney, was removed from a rabbit immediately after death, and was perfused with oxygenated Ringer's solution (containing ·9 % NaCl, ·024 % $CaCl_2$, ·042 % KCl, and as a rule, ·01 % $NaHCO_3$) for a quarter of an hour so as to wash out the blood and lymph. Oxygenated saline containing the poison under investigation was then perfused for half an hour, 30 to 90 c.c. of the liquid being run through. Finally the original Ringer's solution was substituted, and was kept perfusing for the next 10 hours at room temperature. This saline was collected under petroleum, drawn off at about two hour intervals, and the gases in weighed amounts of it (generally about 50 grams) were boiled off *in vacuo*, and analysed. At least two samples of the saline solution before perfusion were similarly analysed, and a mean taken. By comparing the change in the composition of the gases before and after perfusion, both the CO_2 output and the oxygen intake were determined.

In the above mentioned papers I showed that when a kidney is perfused continuously with oxygenated saline, its gaseous metabolism rapidly falls, till after 12 hours it becomes reduced to about two-fifths its original amount. This loss of respiratory power is invariably observed in all kidneys perfused with saline, and hence it can be allowed for. The average curve of CO_2 output of normal unpoisoned kidneys is given as a dotted line in all figures in this paper. If the perfusion of

[1] T. B. Robertson. *Journ. Biol. Chem.* I. p. 507. 1906.

[2] Vernon. This *Journal*, XXXV. p. 53. 1906; XXXVI. p. 81. 1907.

normal kidneys is made with saline containing 1 % of rabbit's serum, the gaseous metabolism is reduced by only about a fourth in the 12 hours. The average curve of CO_2 output under these conditions is likewise given as a dotted line in a few cases (Figs. 8, 15 and 18).

The action of alkalis upon tissue respiration.

It will be convenient to discuss first those poisons which probably form easily dissociable compounds with certain constituents of the protoplasm of the tissues. Besides salts, they include acids and alkalis. It was found to be impossible to investigate the action of the powerful caustic alkalis upon tissue respiration, as an attempt to perfuse a kidney with saline containing ·005 % of caustic soda led to such marked vaso-constriction that after a few minutes the flow of saline entirely stopped. With a ·003 % solution it was so slow that insufficient saline was obtainable for analysis, but a ·001 % solution perfused well at maximum pressure (150 cm. of water). However at this dilution the alkali seemed to be without effect on the respiration.

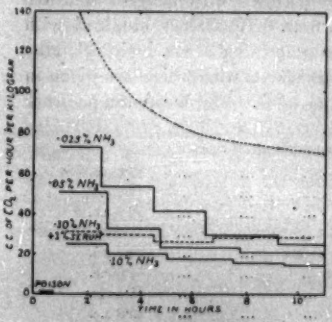

Fig. 1. Ammonia.

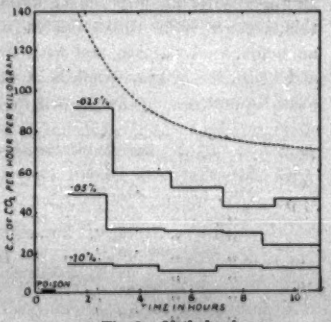

Fig. 2. Methylamine.

In the case of ammonia, the action of Ringer's solution containing ·025, ·05 and ·1 % of the poison was investigated, and from the curves in Fig. 1 it will be seen that the effect produced was roughly proportionate to the concentration. These curves indicate the volume of CO_2 evolved per hour per kilogram of kidney tissue. In each experiment the gaseous metabolism of the kidney gradually dwindled down during the course of the 11 hours' perfusion, as it does in unpoisoned kidneys. In

the experiment represented by the dotted line curve near the bottom of the figure, the perfusion liquid, both during the half hour's poisoning and subsequently, contained 1 % of serum. This to some slight extent protected the kidney tissue from the ammonia, and also it prevented the gaseous metabolism of the kidney dwindling so rapidly during the course of the subsequent 10 hours' perfusion.

A similar series of experiments was made with methylamine, and it will be seen that the effects produced by the three concentrations closely resemble those produced by the corresponding concentrations of ammonia. In all the experiments with methylamine, and in those with ·1 % of ammonia, the saline solution used for perfusion contained ·01 % $NaHCO_3$, whilst in those with ·025 and ·05 % of ammonia, no $NaHCO_3$ at all was added. The presence or absence of this salt in these particular experiments probably made no difference, but as will be seen later on, this is by no means always the case.

To obtain a numerical measure of the effects produced, I have calculated the average CO_2 output per hour per kilogram from 1½ hours after the beginning of the experiment up to the end of the 11th hour. In the case of an unpoisoned kidney perfused with Ringer's solution, this average value works out at 87·6 c.c. The values obtained with ammonia, methylamine, and with two other alkalis, viz. hydroxylamine and hydrazine hydrate (details of experiments with which are given in a subsequent section) are shown in the table. The mean temperature

Mean CO_2 output per hour between 1½–11 hours.

Normal unpoisoned kidney	87·6 c.c.
After ½ hr. of ·025 % NH_3	41·2 c.c.
,, ,, ·05 % ,, ...	26·9 c.c.
,, ,, ·10 % ,, ...	18·3 c.c.
,, ,, ·10 % ,, + 1 % serum	28·8 c.c.
,, ,, ·025 % methylamine	57·5 c.c.
,, ,, ·05 % ,, ...	31·4 c.c.
,, ,, ·10 % ,, ...	12·8 c.c.
,, ,, ·10 % hydrazine	67·8 c.c.
,, ,, ·20 % ,, ...	55·8 c.c.
,, ,, ·05 % hydroxylamine	55·2 c.c.
,, ,, ·10 % ,, ...	56·4 c.c.

of perfusion in the ammonia and methylamine experiments varied from 15·4° to 16·9°, and in the other experiments, from 17·0° to 17·8°, so the data are roughly comparable. We may say that on an average similar concentrations of ammonia and of methylamine have similar effects, and that ·20 % of hydrazine hydrate has about the same effect as ·025 %

of methylamine or of ammonia. Or, calculated according to their molecular weights, we may say that $\frac{N}{25}$ hydrazine hydrate, $\frac{N}{68}$ ammonia, and $\frac{N}{124}$ methylamine, have similar destructive effects upon the tissue respiration. From the electrical conductivity values of solutions of these bases determined by Bredig[1], one can calculate that at these dilutions the conductivity varied as about 1 : 7 : 27. That is to say, the more ionised the base, the greater is its poisonous influence. As far as these very imperfect data can show, the poisonous effect is proportional to the square root of the electrical conductivity. Thus the ratio of the toxically equivalent solutions of hydrazine hydrate, ammonia and methylamine is as 1 : 2·7 : 5, whilst the ratio of the square roots of their electrical conductivities is as 1 : 2·6 : 5·2.

In the case of hydroxylamine the conductivity values have not been determined, as the substance is so unstable in dilute solution. It quickly splits up into ammonia and nitrogen, and hence the irregular values given in the above table may be due to a variable amount of such decomposition. In any case hydroxylamine is known to be a much weaker base than ammonia, and we see that it likewise acts much less harmfully on tissue respiration.

In that half an hour's exposure to the action of dilute ammonia causes so much destruction of tissue respiration, it naturally follows that continuous perfusion with saline containing ammonia leads to more rapid destruction still. In a former paper the effects of ·005 to ·025 % ammonia solution are described, and these results show that with ·01 % or more of NH_3 the CO_2 output was diminished to 4 c.c. or less per hour after about nine hours of continuous perfusion.

The action of acids.

The action of only two acids was investigated, one organic and one inorganic. The results obtained with (inactive) lactic acid are shown in Fig. 3. They do not form a regular series as in the above recorded experiments with alkalis, for whilst ·2 % lactic acid had comparatively little influence on the respiratory powers of the kidney tissue, ·3 and ·4 % had very marked effects. In the experiments with ·2 and ·4 % acid, the perfusion saline contained ·01 % $NaHCO_3$, whilst in that with ·3 % acid, it contained none. The absence of sodium bicarbonate in

[1] Bredig. *Zeit. phys. Chem.* XIII. p. 308. 1894.

this experiment is probably to be held accountable for the gradual diminution in the CO_2 output of the kidney during the course of the experiment, for when a kidney was poisoned with saline containing ·3 % lactic acid, and was then perfused with saline containing ·02 % $NaHCO_3$, its CO_2 output rose considerably during the course of the subsequent perfusion. At first it amounted to only 11·6 c.c. per hour, but by the 7¼ to 9¼ hour period of perfusion it had risen to 29 c.c. per hour. Again, when a kidney was poisoned for half an hour with a saline solution containing ·4 % lactic acid, ·01 % $NaHCO_3$ and 1 % serum, and was subsequently perfused with the same solution, less the lactic acid, its CO_2 output at first amounted to 15·5 c.c. per hour, and then gradually rose till in the 8¼—10 hour period it amounted to 25 c.c. per hour. For the sake of clearness, both of these curves are reproduced in the upper part of the figure.

Even in the experiment in which a kidney was poisoned with ·4 % lactic acid, and was then perfused with saline containing ·01 % $NaHCO_3$, there was a slight rise of CO_2 output during the course of the subsequent perfusion. Hence these results with lactic acid differ greatly from those obtained with alkalis, for in their case the CO_2 output invariably diminished during the course of perfusion. The poisonous effect of an alkali is therefore more permanent, and seems to admit of no recovery of respiratory power on the part of the damaged tissues.

As will be pointed out in a subsequent section of this paper, the respiratory quotient of the kidney is uninfluenced by the majority of poisons used, but this is not so in the case of acids. The table gives

Concentration of lactic acid	Mean temp. of perfusion	Respiratory quotients	Oxygen intake in c.c. per hour per kilogram
·20 %	15·7°	*·91*, ·84, ·76, ·82	77, 82, 83, 75
·30	16·5	·41, ·61, *·90*, ·94, *·98*	28, 36, 24, 30, 29
·30	16·5	*·68*, ·67, *·67*, ·69, ·67	34, 29, 29, 27, 26
·40	16·7	*·1·05*, *·71*, *·90*, *1·05*, *1·23*	19, 26, 21, 20, 18
·40 + 1 % serum	16·8	*·42*, *·60*, ·66, *·70*, 76	37, 31, 33, 33, 34

the quotients observed in the successive perfusion periods indicated in each of the curves in Fig. 3. As will be described later, the values in italics have been corrected for an experimental error, and hence are not quite so reliable as the uncorrected values. But taking the values as they stand, it will be seen that in two experiments the quotients were abnormally low at first, and gradually rose during the course of perfusion to a more or less normal value. Turning to the oxygen intake values

given in the right half of the table, it will be seen that in these two experiments they do not show any definite rise like the CO_2 output values. That is to say, the lactic acid acted more powerfully upon the CO_2-producing mechanism of the kidneys than upon their oxygen-absorbing mechanism, but this extra action was only a temporary one from which the tissues gradually recovered in the course of perfusion.

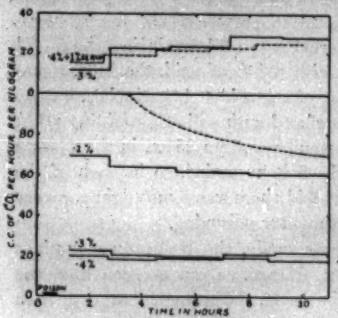

Fig. 3. Lactic acid.

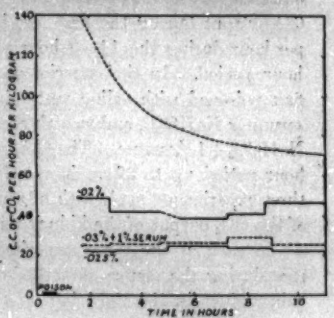

Fig. 4. Nitrous acid.

In a previous paper I showed that if a kidney were perfused continuously for 11 hours with saline containing ·06 to ·20% of lactic acid, its CO_2 output was gradually lowered till in the ·20% acid experiment it had fallen practically to zero, and in the other experiments, to from 4 to 10 c.c. per hour. But the oxygen intake was by no means equally affected, for the respiratory quotient was gradually depressed to a value of ·38 to ·46, or by a greater amount than that effected by stronger acid acting for half an hour.

For reasons explained later on, the inorganic acid employed in these experiments was nitrous acid, or rather a mixture of nitrous and nitric acids containing an unknown amount of nitrous acid. The acid was prepared by heating sodium nitrite with nitric acid of Sp. G. 1·35, and condensing the nitrous fumes in water. The dilute solution thereby obtained undoubtedly contained some nitrous acid, as it continued to evolve NO_2 for some days after it was used. The effect of saline containing ·02 to ·03% of this mixture of acids and ·1% of sodium nitrite (but no $NaHCO_3$) was investigated. In the experiment represented by the uppermost curve of Fig. 4, the kidney was subsequently perfused for the first two periods with saline containing no $NaHCO_3$.

Then saline containing ·02 % $NaHCO_3$ was substituted, and 45 minutes later, by which time the alkali-less saline was almost washed out of the kidney, samples of the perfused saline were again collected. They show a gradual though slight rise of CO_2 output as perfusion proceeded. In the experiment with ·025 % of acid, the kidney was perfused with saline containing ·03 % $NaHCO_3$ from the end of the half hour's poisoning onwards, and it will be seen that there was a slight but distinct rise of CO_2 output during the first few hours of perfusion, viz. from 22·4 c.c. per hour during the 1¼—3 hour period up to 27 c.c. during the 5—7¼ hour period. In the experiment with ·03 % of acid, the kidney was first poisoned with saline containing ·03 % acid + ·1 % $NaNO_2$, but no serum or $NaHCO_3$, and was then perfused with saline containing ·01 % $NaHCO_3$ + 1 % serum. The CO_2 output rose from 25 c.c. in the 1¾—2¾ hour period up to 29 c.c. in the 7¼—9 hour period, so in each of the three experiments made with nitrous acid there was some slight recovery of the CO_2 output of the kidney tissue after poisoning.

In each of the three experiments made, the respiratory quotient rose during the course of perfusion. Hence, as can be seen from the values given in the right half of the table, the oxygen intake in each

Concentration of nitrous acid	Mean temp. of perfusion	Respiratory quotients	Oxygen intake in c.c. per hour per kilogram
·02 %	16·8°	·58, ·80, — ·90, ·75	79, 53, 47, 46, 66
·025	16·6	·72, ·85, 1·04, ·94, ·85	31, 26, 26, 27, 24
·03	16·3	·47, ·70, ·88, ·87, ·84	54, 37, 31, 34, 31

case gradually fell. The missing quotient in the first experiment is due to the change of perfusion saline from one containing no $NaHCO_3$ to one containing ·02 % $NaHCO_3$. In such a case the CO_2 output of the kidney cannot be determined for the next hour or more, until complete equilibrium has been attained, and hence the CO_2 value given in the curve in Fig. 4 is the oxygen intake value × ·85 (the average quotient).

As far as the few results obtained can show, it appears that ·025 to ·3 % of nitrous + nitric acids had rather less toxic effect on the kidney respiration than ·3 to ·4 % of lactic acid. Probably the inorganic acid is about ten times more toxic than the organic acid. No conductivity determinations for nitrous acid exist, but at a dilution of ·03 % $\left(\frac{N}{157}\right)$ nitric acid[1] has a conductivity 15 times greater than that of ·3 % lactic acid $\left(\frac{N}{80}\right)$, and probably nitrous acid is not much inferior to nitric acid.

[1] Ostwald. *Allgem. Chem.* III. 1. p. 727. Leipzig, 1893: *Zeit. f. physik. Chem.* III. p. 191. 1889.

Arguing on these doubtful premisses, therefore, it would seem that the toxicity of these two acids is roughly proportional to their electrical conductivity.

The action of mercuric chloride.

As a type of heavy metal salts, mercuric chloride was chosen, and saline containing ·001 to ·4 % of $HgCl_2$ was perfused for half an hour. Some of the results obtained are shown in Fig. 5. The most dilute solution of the sublimate (1 in 100,000), though it reduced the initial respiratory power of the kidney tissue to about half the normal, had apparently no effect whatever on the respiration during the last few hours of perfusion. The other concentrations of sublimate, though they varied as 1 to 40, had almost the same effect upon the respiration. Of the curves given in the figure, it will be seen that those for ·01 % and for ·03 % almost coincide, whilst a curve obtained with ·1 % $HgCl_2$ was almost identical with them, but is not introduced for the sake of clearness. Again we see that the curves for ·1 and ·4 % $HgCl_2$ almost coincide, whilst another obtained with ·03 % $HgCl_2$, and not reproduced, was almost identical with them. The non-dependence of toxic effect upon concentration is best shown by the following data, which indicate the average output of CO_2 per hour during $1\frac{1}{2}$ to 11 hours of perfusion.

Concentration of $HgCl_2$	Perfusion liquid	Average CO_2 output
·001 %	Ringer	81·6 c.c. per hour
·01	,, +2 % serum	44·4 ,,
·03	,, +1 % ,,	26·5 ,,
·08	,,	46·2 ,,
·10	,, +1 % ,,	54·9 ,,
·10	,,	25·6 ,,
·40	,,	23·3 ,,

In three out of the seven experiments the kidney, after the initial poisoning with Ringer's solution + the sublimate, was perfused with saline containing 1 or 2 % of serum. In the experiment with ·1 % $HgCl_2$ this apparently provoked a larger CO_2 output, but in that with ·03 % $HgCl_2$ there was a much smaller output than in the absence of serum. Hence one cannot conclude that it had any influence whatever. The great irregularity of the results must be due to individual differences in the kidneys, as the temperature and other conditions of perfusion were nearly constant.

These results differ in a striking manner from those obtained with acids and alkalis, as they show that above a certain minimal limit the

poisonous influence of the mercuric chloride is very little affected by the concentration. Presumably a certain definite proportion of the poison is abstracted from the perfusion liquid and becomes bound up in the tissues, whilst the remainder passes on, unattracted by the tissues, and therefore with very little influence upon them. In that perfusion with ·01 % $HgCl_2$ produced as much destruction of respiratory power as perfusion with ·03 or ·1 % $HgCl_2$ (in some cases), it might be argued that this represents the maximum toxic concentration. In the actual experiment only 30 c.c. of this ·01 % solution were perfused, and hence at most ·003 gm. could have been absorbed. The kidney of the rabbit contains on an average 16·5 % of protein[1], and as the kidney used in this experiment weighed 7·25 gm., it contained 1·20 gm. protein, or 400 times the maximum weight of $HgCl_2$ absorbed. Hence a molecular weight of $HgCl_2$, or 271 parts, might be combined with 10,840 parts of protein, or about twice what is usually regarded as the minimum molecular weight of a protein.

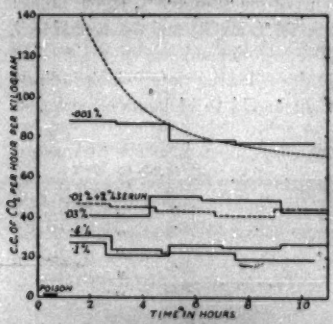

Fig. 5. Mercuric chloride.

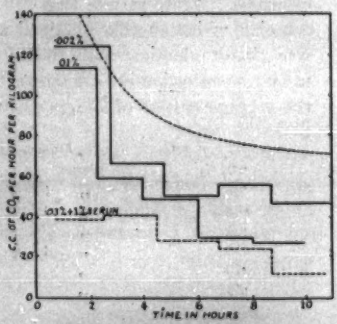

Fig. 6. Mercuric chloride.

These experiments with corrosive sublimate are interesting as showing that even when the tissues are saturated with the salt, some respiratory power is still retained. It seems likely, therefore, that the chemical groupings in the tissues which are specially concerned in tissue respiration are not directly affected by the mercury salt, but are injured indirectly by its fixation to other tissue constituents.

A few experiments were made in which the kidney was perfused continuously for 11 hours with saline containing mercuric chloride.

[1] Vernon, *Zeit. f. allgem. Physiol.* VI. p. 393. 1907.

From the curves in Fig. 6 it will be seen that ·002 % $HgCl_2$ caused a rapid deterioration of respiratory power for the first $2\frac{3}{4}$ hours, whilst from the 5th hour onwards the CO_2 output remained nearly constant. Previous to this constant period, the tissues were presumably absorbing and fixing the mercuric salt from the perfusion liquid till they reached their saturation point. During the first $2\frac{3}{4}$ hours 160 c.c. of saline, containing ·0032 gm. of $HgCl_2$, were perfused, and probably a small amount more of the salt was fixed in the $2\frac{3}{4}$—$4\frac{3}{4}$ hour perfusion period. The kidney weighed 8·3 gm., hence the total amount absorbed was probably very similar to that absorbed in the above mentioned experiment.

In the next curve in Fig. 6 the perfusion saline contained ·01 % $HgCl_2$, and the CO_2 output diminished rapidly for the first six hours of perfusion, and then more slowly. In the lowest curve, saline containing 1 % of serum and ·03 % $HgCl_2$ was used, and here again the gaseous metabolism continued to diminish throughout the experiment. Probably, therefore, the excess of $HgCl_2$ over and above that fixed by the tissues continued to exert a slow destructive effect upon the tissue respiration. Even during half an hour's perfusion this is slightly the case, for we saw in the previous results that on an average the CO_2 output was rather less after perfusion with ·1 and ·4 % $HgCl_2$ than after ·01 and 03 %. When the perfusion with the mercury salt is continuous, however, the destructive effect is naturally more marked unless a very small amount of the salt, such as ·002 %, is present.

It should be mentioned that the compounds of protein with mercuric chloride, though insoluble under ordinary conditions, are dissolved by excess of protein and by sodium chloride solutions, so the perfusion liquid used in this last experiment was free from any precipitate.

The action of acid sodium sulphite.

As long ago as 1880 O. Loew[1] suggested that the lability of living protoplasm is due to its containing both aldehyde and amido groupings, for such substances as readily act upon these groupings are likewise protoplasmic poisons. For instance hydroxylamine, hydrazine, phenyl hydrazine, hydrocyanic acid and sulphuretted hydrogen readily react with aldehydes, whilst cyanogen, nitrous acid and formaldehyde react with amido groups. In that the former class of bodies has no action on

[1] O. Loew. *Die chemische Energie der lebenden Zellen.* 2nd ed. Stuttgart. 1906.

dead protoplasm, it follows that it contains no aldehyde groupings, or that such groupings disappear at the time of death.

It must be admitted, however, that some of the evidence adduced by Loew does not support his hypothesis. Hydrocyanic acid, though extremely poisonous for mammals, is not so for phanerogams, algæ and bacteria. Thus ·1 % HCN takes 15 hours to kill them, whilst ·05 % HCN takes some hours to kill infusoria. Hence aldehyde groupings, if present in these lower organisms, must be less sensitive or of less vital importance than those in warm-blooded animals. Again, a ·05 % solution of formaldehyde takes two hours to kill crustacea, worms and molluscs, whilst ·1 % takes six days to kill phanerogams. A dose of ·24 gm. per kgm. is sufficient to kill a rabbit.

One of the chief objects of the present research was to test the validity of Loew's hypothesis in its application to the respiratory powers of living tissues, for one striking piece of evidence already existed in its support. In 1889 Geppert[1] studied very fully the action of non-lethal doses of hydrocyanic acid upon the respiratory exchange of mammals, and he found that the oxygen absorbed by the animal was much diminished, even during the powerful convulsions produced by the poison. The tissues had lost much of the power of absorbing oxygen, though they regained it after a time as the hydrocyanic acid became broken down into other products and removed. In a previous paper I showed that after perfusing kidneys for half an hour with saline containing ·1 to ·2 % of HCN, their gaseous metabolism was greatly reduced, but that on subsequent perfusion with oxygenated saline they gradually recovered some of it. In fact the kidney poisoned with ·1 % HCN attained the CO_2 output of normal kidneys after nine hours' perfusion.

Again, Schröder[2] showed that if *Aspergillus niger* were poisoned with KCN solution, its oxygen absorption was greatly diminished, whilst its CO_2 output was practically stopped. If the KCN were washed away from the fungus, it recovered its respiratory powers in one to four hours.

Though there can be no doubt that the tissues possess the power of loosely binding hydrocyanic acid, there is no proof that they do so in virtue of the presence of aldehyde groupings, for this acid can enter into many different forms of combination. A much more typical and distinctive substance with which aldehydes react is an acid sulphite.

[1] Geppert. *Zeit. f. klin. Med.* xv. p. 398. 1889.
[2] Schröder. *Jahrb. f. wiss. Bot.* xliv. p. 409.

Benzoic aldehyde, for instance, readily combines with acid sodium sulphite to form an additive compound which is decomposed by alkaline carbonates, and acetaldehyde combines almost as readily. Hence the poisonous effects of this sulphite salt upon tissue respiration were investigated at some length. The acid sulphite used was prepared by saturating a solution containing a known amount of Na_2SO_3 with SO_2, estimating the total SO_2 present in a known volume of it, and adding sufficient Na_2SO_3 to the SO_2-saturated solution to combine with all the SO_2 over and above that already present as $NaHSO_3$.

In Fig. 7 are shown the results obtained on poisoning kidneys for half an hour with saline containing ·05 to ·20 % $NaHSO_3$, and then perfusing them for the next 10 hours with saline containing CaCl, and KCl, but no $NaHCO_3$. During the first hour or two of perfusion appreciable amounts of sulphite continued to break away from the tissues, and as the SO_2 was liberated on boiling the perfusion liquid *in vacuo* with dilute acid, it ranks as CO_2 in the analysis. Hence in all cases the respiratory quotient obtained during the first perfusion period, and sometimes that in the second period, was too high. The oxygen values are unaffected by this error, so all the curves in Figs. 7 and 8, with one exception, represent the volumes of oxygen absorbed by the kidneys, and not the volumes of CO_2 excreted. The oxygen estimation is not so reliable as the CO_2 estimation. Still, the values given in these figures correspond closely with the CO_2 values obtained for all but the first perfusion period. The dotted line curve representing the gaseous metabolism of normal kidneys shows their oxygen intake, *i.e.* the CO_2 output divided by the average quotient of ·85.

To return to the curves in Fig. 7, it will be seen that the acid sulphite destroyed a large part of the respiratory capacity of the tissues, ·15 and ·20 % of the salt being apparently much more poisonous than ·05 and ·10 %. In three out of the four experiments the gaseous metabolism distinctly dwindled down during the course of the prolonged perfusion, whilst in the experiment with ·20 % $NaHSO_3$ it showed a slight rise.

Very different from these results are those obtained when the poisoned kidney is perfused with saline containing sodium bicarbonate, as can be seen from the curves in Fig. 8. In all these experiments the kidneys were first poisoned with ·2 to ·25 % $NaHSO_3$. In the case of the lowest curve given in the figure, the saline used for subsequent perfusion contained ·01 % $NaHCO_3$. The values of this particular curve (except the value for the first perfusion period, which is the

oxygen value × ·85) represent the CO_2 output values, and it will be seen that they indicate a slight increase of respiration for the first nine hours of perfusion. The oxygen intake in this experiment remained practically constant throughout at 27 to 33 c.c. per hour, or differed but little from the oxygen intake shown in the two lowest curves of Fig. 7. Omitting for the moment the next two lowest curves in Fig. 8, we come to a curve showing the effect of perfusion with saline containing ·02 % $NaHCO_3$. In its case the oxygen intake rose gradually from 52 c.c. per

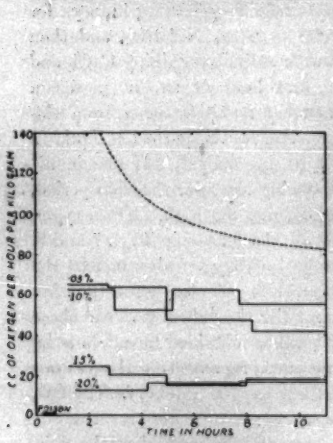

Fig. 7. Acid sodium sulphite.

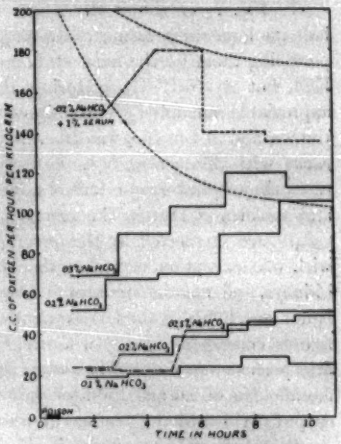

Fig. 8. Acid sodium sulphite.

hour up to a maximum of 105 c.c. per hour in the 9—11 hour period of perfusion. This maximum, be it noted, corresponds almost exactly with the oxygen intake of normal kidneys perfused with saline containing ·02 % $NaHCO_3$, or apparently the poisoning with $NaHSO_3$, though it temporarily reduced the gaseous metabolism to a third the normal, had no permanently injurious effect whatever upon it. It should be mentioned that the gaseous metabolism of normal kidneys perfused with saline containing ·02 or ·03 % $NaHCO_3$ is slightly higher than when saline containing ·01 % $NaHCO_3$ is used[1].

The next curve in the figure shows the effect of perfusion with saline containing ·03 % $NaHCO_3$. In this case the initial oxygen intake

[1] Vernon. This *Journal*, xxxvi. p. 81. 1907.

is larger (viz. 70 c.c. per hour), and it increases more rapidly during the course of perfusion, so that it attains its maximum of 120 c.c. per hour in the 7—9½ hour period. In the experiment represented by the uppermost curve, the kidney was poisoned with saline containing 1 % serum, ·02 % $NaHCO_3$, and ·25 % $NaHSO_3$, and was then perfused with fluid of the same composition, less the acid sulphite. Probably the kidney reached its maximum oxygen intake in the 4½—6½ hour perfusion period, but it is not possible to speak for certain, as during the first hour of the 2½—4½ hour period the perfusion almost ceased owing to insufficient pressure, and hence the oxygen intake value obtained (100 c.c. per hour) was much too low.

It is evident, from these results, that the recovery of the respiratory power of the kidney tissue after poisoning with acid sodium sulphite depends on the action of the alkaline bicarbonate present in the perfusing saline. A more striking proof of this is afforded by the lowest curve but one in the figure. In this experiment the kidney, after poisoning with ·2 % $NaHSO_3$, was perfused for the next 4½ hours with saline containing no $NaHCO_3$. Its oxygen intake accordingly remained constant at about 21 c.c. per hour. Saline containing ·025 % $NaHCO_3$ was then substituted, and immediately the oxygen intake began to rise, till in the 9½—11 hour period it was 50 c.c. per hour. In the next curve shown in the figure the kidney, after poisoning with ·25 % $NaHSO_3$, was perfused with saline containing no $NaHCO_3$ for the first perfusion period only. On substituting saline containing ·02 % $NaHCO_3$ the oxygen intake gradually rose from its original value of 22 c.c. per hour up to a maximum of 51 c.c. per hour in the 9—11 hour period.

These experiments clearly suggest, therefore, that the acid sulphite enters into combination with certain constituents of the tissues and thereby prevents them from exerting their normal respiratory powers. The alkaline carbonate of the perfusion saline, in proportion to its concentration, has the power of slowly dissolving this bond; the acid sulphite breaks away from the tissues, and they regain their normal respiratory powers. It will be remembered that in all the experiments represented in Fig. 8 the kidneys were poisoned with about the same strength of acid sulphite, or their respiratory powers, immediately after the poisoning, must have been about the same. Yet we see that the oxygen intake during the first period measured was very different. This is chiefly because it is useless to collect and analyse the saline perfusing through the kidney during the first half hour after the poisoning, as equilibrium of conditions as regards rate of perfusion, diffusion of gases from the surface

of the previously exposed kidney and from the supernatant petroleum into the perfused saline, etc. has not been attained. By the end of this half hour kidneys perfused with saline containing ·02 or ·03 % $NaHCO_3$ have already recovered a good deal of their respiratory powers, as can be judged from the general contour of the curves in Fig. 8. The kidneys perfused with the alkaline saline after an initial $1\frac{1}{2}$ or $4\frac{1}{2}$ hours of alkali-less saline did not recover nearly so much respiratory power as the kidneys perfused with the alkaline saline from the outset, probably because the acid sulphite retained in the tissues for this longer period acted destructively upon them.

The action of sodium fluoride.

Some aldehydes form combinations with inorganic salts such as fluorides. For instance benzoic aldehyde forms the compound $C_7H_6O.BF_3$ with boron fluoride. There is very little doubt that fluorides likewise enter into combination with some constituent of living tissues, whether aldehyde or some other grouping, and thereby temporarily deprive them of their respiratory powers. In the experiments to be described the kidney was poisoned for half an hour as usual by perfusing with a solution containing ·1 to 1·5 % of NaF, ·01 % $NaHCO_3$, and in some cases, small quantities of other salts and of serum. But such additions seemed to make no difference. In fact, the curves given in Fig. 9, except that in which 1·5 % NaF was used, are almost identical. In every case the CO_2 output was at a minimum during the first perfusion period, gradually rose during the next few hours till it became equal to that of normal unpoisoned kidneys, and then gradually sank again at about the rate shown by normal kidneys. The actual CO_2 output during the first period varied only from 43 to 52 c.c. per hour: that during the third or maximum period varied from 77 to 94 c.c. per hour, and that during the final period from 58 to 72 c.c. per hour. Poisoning with 1 % NaF instead of ·1 % made no difference whatever to the subsequent respiration, but when 1·5 % NaF was used the initial CO_2 output was lowered to 14 c.c. per hour, and subsequently rose to a maximum of only 43 c.c. per hour in the $4\frac{1}{2}$ to $6\frac{1}{2}$ hour perfusion period. Perhaps this is not altogether a genuine result, as the sodium fluoride of commerce is frequently not neutral, and though it was carefully neutralised before use it is possible that exact neutrality was not always attained, and that the destructive effect was partly due to the slight excess of acid or alkali present. The several experiments

on the action of sodium fluoride were made at different times with different samples of the salt.

In the two experiments reproduced in the figure as dotted lines 1 % of serum was added to the sodium fluoride solution used for poisoning the kidney, and to the saline used in the subsequent perfusion, but this made little if any difference to the gaseous metabolism.

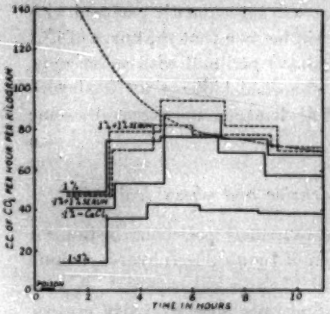

Fig. 9. Sodium fluoride.

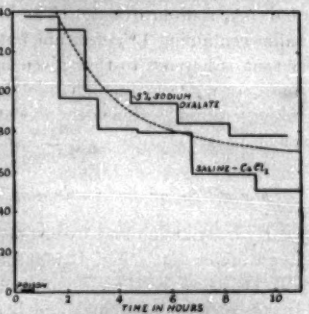

Fig. 10. Sodium oxalate.

When ·1 % NaF was used for poisoning, it was dissolved in ·9 % NaCl, and in the one case ·042 % KCl + ·024 % $CaCl_2$ was added, as well as 1 % serum. In the other case no $CaCl_2$ was added, whilst the saline used during the subsequent 10 hours' perfusion was likewise calcium-free. It contained only ·9 % NaCl, ·042 % KCl and ·01 % $NaHCO_3$. The object of this experiment was to see if the absence of calcium salts in any way retarded the recovery of respiratory power by the fluoride-poisoned kidney. Thus calcium forms an insoluble compound with fluorides, and the removal of the fluoride from the tissues might have been due to the calcium present in the perfusion liquid. This was evidently not the case, and the absence of calcium from the perfusion liquid seemed to be entirely without effect on the respiration. In Fig. 10 the lower curve shows the effect of perfusion of a normal unpoisoned kidney with calcium-free saline, and it will be seen that it differs very little from the normal curve. Such difference as exists is probably due to temperature, as the normal curve was obtained at 19·0° C., and the calcium-free one at 16·4°.

This single experiment, as far as it goes, seems to indicate that calcium salts are unnecessary for the maintenance of tissue respiration,

a result in disaccord with the well-known experiments of Ringer[1] upon the action of calcium-free saline upon the beat of the frog's heart. Presumably the duration of the experiment was insufficient to reveal the importance of calcium salts. Another experimental result, perhaps bearing on the point, is also shown in Fig. 10. The upper curve gives the CO_2 output of a kidney which was poisoned for half an hour with saline containing ·5 % NaCl, ·042 % KCl, ·01 % $NaHCO_3$, 1 % serum and ·5 % sodium oxalate. The kidney was subsequently perfused with saline containing 1 % of serum, but it will be seen that the curve of CO_2 output is lowered to that given by kidneys perfused with saline only, and does not remain high like that of normal kidneys perfused with saline + serum. Hence the oxalate had damaged the tissues in some way.

The action of hydroxylamine, hydrazine and phenyl-hydrazine.

Hydroxylamine and hydrazine are extremely poisonous bodies, for Loew states that a 1 in 10,000 solution of (neutralised) hydroxylamine kills crustacea in three hours, and will likewise kill bacteria. A 1 in 10,000 solution of neutralised hydrazine kills algæ in a day or two, whilst a 1 in 5000 solution kills bacteria. The free bases are probably much more poisonous still. Free hydroxylamine readily combines with aldehydes to form aldoxims, $R.CH=NOH$, whilst free hydrazine with difficulty combines to form extremely unstable hydrazones. Phenyl-hydrazine combines so readily with aldehyde that if phenyl-hydrazine hydrochloride and sodium acetate are added to a solution containing 1 in 50,000 of benzaldehyde, a white precipitate of phenyl-hydrazide is produced. Hence if Loew's hypothesis as to the existence of aldehyde groupings in living tissues is valid, one would expect to obtain some evidence in support of it by experiments with these poisons.

Fig. 11 represents the results obtained with hydroxylamine. A solution of the free base was prepared shortly before each experiment by precipitating hydroxylamine sulphate with slight defect of baryta. A kidney was poisoned for half an hour with ·05 % NH_2OH, and was then perfused with saline containing no $NaHCO_3$, whilst another kidney was poisoned with ·1 % NH_2OH, and was then perfused with saline containing ·01 % $NaHCO_3$. The CO_2 output of this kidney did not fall off so rapidly during the course of perfusion as that of the other kidney, but in neither case was there any indication of recovery of respiratory power

[1] Ringer. This *Journal*, IV. p. 29. 1883.

as in kidneys poisoned with $NaHSO_3$ or NaF. In fact, the curves are very similar to those obtained with kidneys poisoned with ammonia. The lowest curve in the figure represents the CO_2 output of a kidney perfused continuously with saline containing ·025 % NH_4OH. The values given are in reality the oxygen intake values × ·85, for hydroxylamine is such an unstable substance that it partly decomposes into N_2O and other gases during perfusion through the kidney or in the subsequent boiling off of the gases from the saline, and thereby sent up the respiratory quotients to 1·1—1·3. By comparing this curve with those given in a previous paper, it will be found that the gaseous metabolism dwindled down at about the same rate as in a kidney perfused with ·005 % of free ammonia.

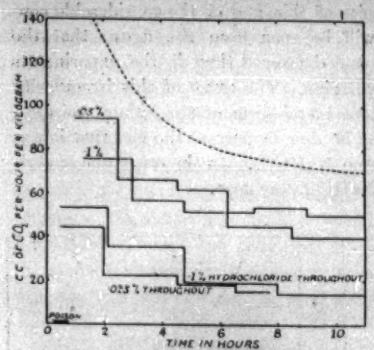

Fig. 11. Hydroxylamine.

The remaining curve in the figure shows the effect of continuous perfusion with saline containing ·1 % of hydroxylamine hydrochloride, but no $NaHCO_3$. The diminution of CO_2 output is greater than in a corresponding experiment in which saline containing ·1 % of ammonium chloride was used, for in this case the CO_2 output was 95 c.c. per hour in the ½ to 2 hour period of perfusion, 74 c.c. in the 2 to 4½ hour period, 48 c.c. in the 4½ to 8 hour period, and 45 c.c. in the 8 to 11 hour period. As far as these very limited experiments can show, therefore, a hydroxylamine salt acts more destructively on tissue respiration than an ammonium salt, though free hydroxylamine acts much less destructively than free ammonia.

Fig. 12 shows the effect of poisoning with saline containing free hydrazine hydrate, $NH_2.NH_2OH$. After half an hour of ·1 % hydrazine, the kidney was perfused with saline containing no sodium bicarbonate, whilst another kidney, after poisoning with ·2 % hydrazine, was perfused with saline containing ·02 % $NaHCO_3$. In neither case was there any indication of recovery of respiratory power during perfusion.

Fig. 13 shows the effects of poisoning with phenyl-hydrazine.

Kidneys were poisoned with saline containing ·1 to ·3 % of phenyl-hydrazine hydrochloride, together with ·15 to ·45 % of sodium acetate. In the experiment with ·3 % phenyl-hydrazine + ·45 % acetate, the saline used contained ·8 % NaCl instead of ·9 %, but its osmotic pressure must still have been too high. In spite of this, and of the considerable concentration of the poison, it will be seen from the figure that the respiration of the kidney was less depressed than in the experiments with ·1 and ·15 % of phenyl-hydrazine. The cause of this irregularity is unknown, but the low gaseous metabolism of the kidney poisoned with ·1 % phenyl-hydrazine may be due in part to the fact that it was perfused with saline containing no $NaHCO_3$. In the other two experiments saline containing ·01 % $NaHCO_3$ was used.

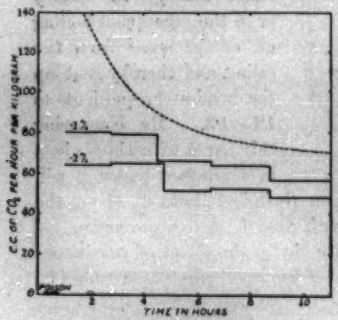

Fig. 12. Hydrazine.

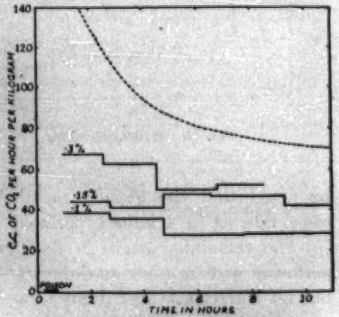

Fig. 13. Phenyl-hydrazine.

Perhaps it would have been possible to have obtained evidence of recovery of respiratory power in kidneys poisoned by hydroxylamine and hydrazines if a wider range of concentrations of the poisons had been used on the one hand, and of alkalinity of perfusion saline on the other. But where so many poisons were available for investigation, it seemed best to repeat observations only with those which gave promising results from the first. Also it is to be remembered that practically speaking it is not possible to use saline containing more than ·03 % $NaHCO_3$, as the error of analysis of the CO_2 output of the kidney becomes so much increased. Again, it is not possible to perfuse with saline containing free acid, though it is known that, for instance, the combination of aldehyde with phenyl-hydrazine is resolved into its components by dilute acids, though not by dilute alkalis. Positive

evidence of the formation of loose combinations of poisons with the tissues, and their subsequent resolution with recovery of respiratory power, is therefore of very much more weight than a much larger volume of negative evidence. When we remember that the presence or absence of ·02 % $NaHCO_3$ in the perfusion liquid was sufficient to determine whether the gaseous metabolism of a kidney poisoned with acid sulphite remained constant or gradually became more than doubled in value, it is evident that it is quite a chance whether the particular conditions of perfusion which lead to recovery are hit off at the first or second trial.

In addition to the group of poisons at present under discussion, it is well known that ammonia and methylamine likewise enter into combination with aldehydes. Still, it is highly improbable that they would have done so under the conditions of poisoning employed. Aldehyde-ammonia, for instance, is prepared by passing ammonia vapour into an ethereal solution of aldehyde, and is a very unstable body, decomposed even by CO_2. Benzaldehyde must be mixed with strong aqueous ammonia, and then combines only very slowly.

The action of urea.

In almost all of the experiments hitherto described, except those with phenyl-hydrazine, the results obtained have been very consistent, and the kidney, though at the outset a living tissue, as a rule responded in its gaseous metabolism to the action of poisons with almost machine-like regularity. However, this was not at all the case in its response to urea poisoning. Urea reacts with aldehydes in cold solution to form diureides, and these bodies are split up into their parent substances by warming with acids. It seemed possible, therefore, that the intimate mechanism of urea excretion by the kidney cells might consist in the annexation of urea molecules from the alkaline blood by aldehyde or other groupings of the kidney cell protoplasm, followed by a liberation of the urea from its combination by the action of the acids secreted in the urine at the same time.

For the sake of clearness, the results obtained with urea are shown in two figures. In all experiments but one the kidney was poisoned for half an hour with a solution containing 2 to 3 % of urea, ·45 to ·225 % of NaCl, ·02 % of KCl, and ·01 % of $CaCl_2$. In the exceptional case it was poisoned for an hour with a solution containing 4 % of urea, ·18 % NaCl, and half the amounts of KCl and $CaCl_2$ mentioned. This more

prolonged poisoning damaged the tissues, and, as can be seen from the lowest curve in Fig. 14, the CO_2 output was less than the normal throughout the perfusion. In all experiments saline containing no $NaHCO_3$ was used for perfusion, and in two of them the saline must have been faintly acid, as small quantities of acid urine were added to it. Thus in the uppermost curve of Fig. 14 ·5 % of (human) urine was added, and in the uppermost curve of Fig. 15, 3 % was added. As pointed out in a previous paper, perfusion with saline containing urine excites the kidney tissues to a slightly increased CO_2 output, and probably the fact that the CO_2 output in the former experiment was slightly above the normal was due to this cause. What the greatly increased metabolism observed in the latter of these two experiments was due to is unexplained. Thus it will be seen that the CO_2 output remained at about 130 c.c. per hour throughout the 11 hours' perfusion, or at a higher value than has been observed in any other case except when unpoisoned kidneys are perfused with saline containing serum.

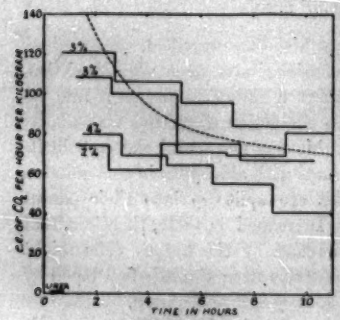

Fig. 14. Urea.

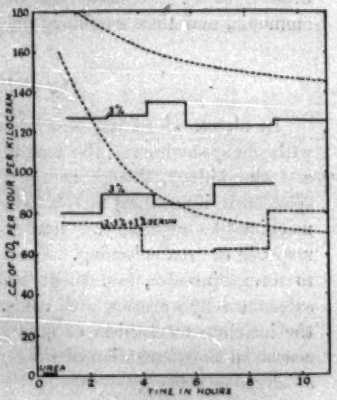

Fig. 15. Urea.

The second curve in Fig. 14 roughly corresponds to the normal, but the third curve, obtained with a kidney poisoned with only 2 % of urea instead of 3 %, shows an abnormally low CO_2 output for the first few hours of perfusion. Then the middle curve in Fig. 15, obtained with a kidney poisoned with 3 % of urea, shows an output of CO_2 rising from 80 c.c. in the first perfusion period up to 94 c.c. in the last period. In

the lowest curve experiment 1 % of serum was added, not only to the saline during the prolonged perfusion, but also to the urea solution during the poisoning, and yet under these presumably very favourable conditions the CO_2 output remained lower than in almost any other experiment.

These experiments therefore show that perfusion of a kidney with 3 % urea solution for half an hour may have little or no destructive effect upon its respiratory powers, but they yield no other definite evidence.

The action of phenol and chloroform.

Though phenol is able to enter into combination with aldehydes and with many other substances, it is doubtful whether it exerts its poisonous action upon living tissues by entering into definite combination with certain of their constituent groupings. Thus it has been suggested by Hans Meyer[1] and by Overton[2] that substances such as chloroform, ether, toluol and phenol act upon protoplasm by first dissolving in the cellular lipoids. They probably act by changing the physical condition of these lipoids, and their poisonous influence is proportional to their active concentration in the blood or lymph surrounding the cell.

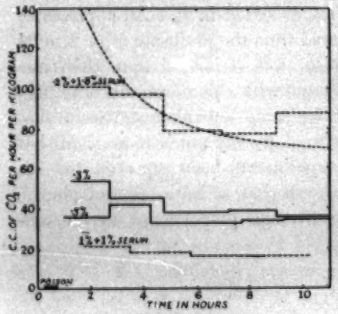

Fig. 16. Phenol.

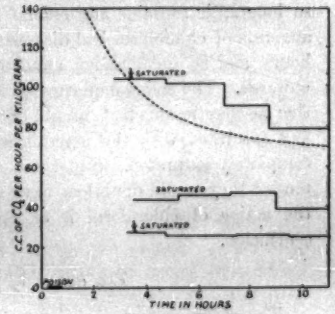

Fig. 17. Chloroform.

The few experiments made with phenol show that, in accordance with theory, it acts destructively upon the gaseous metabolism of the kidney cells in proportion to its concentration. The uppermost curve in

[1] H. Meyer. *Arch. f. exp. Path. u. Pharm.* XLII. p. 109. 1899.
[2] Overton. *Studien über die Narkose.* Jena, 1901.

Fig. 16 was obtained by poisoning a kidney for half an hour with Ringer's solution containing ·2 % of phenol + 1 % of serum, and then perfusing with saline containing 1·8 % of serum. Perhaps this serum is responsible for the slight destruction of respiratory power as compared with that produced by ·3 % of phenol. In this experiment, as in that with ·5 % phenol, no serum was present either during the poisoning or subsequently, but in that with 1 % of phenol it was present throughout. The saline used always contained ·01 % $NaHCO_3$.

A few experiments were made upon the poisonous action of chloroform, but they are not very satisfactory for several reasons. In the first place, one never knows what concentration of chloroform actually acts upon the kidney cells. Three experiments were made, viz. with saline saturated with chloroform immediately before perfusing, with such saline mixed with an equal volume of normal saline, and with such saline mixed with three volumes of normal saline. But saline dissolves such a small amount of chloroform (about 1 part in 300) and the amount of saline perfused in half an hour was so small (45, 52 and 30 c.c. respectively in the three cases mentioned) that the kidney cells could not have become saturated with chloroform up to the amount present in the perfusing saline. A greater objection lies in the fact that it is impossible to analyse the gases in a sample of perfused saline so long as it contains any chloroform dissolved in it. All appreciable amounts of chloroform had disappeared from the perfusate after 2 to 2¼ hours, and so the gaseous metabolism was estimated from this time onwards. The saline a quarter saturated with chloroform did not affect the gaseous metabolism at all, but for some unexplained reason that half saturated with the narcotic injured it very much more than that completely saturated. Had these experiments been repeated and extended they would doubtless have shown that, as in the case of phenol, the action of chloroform is on an average proportional to its concentration.

The action of formaldehyde.

The aldehyde groupings of living tissues, according to Loew, are bound up with amido groupings to form amido-aldehydes, and substances such as nitrous acid and formaldehyde exert their poisonous action by reacting with these amido groups. The experiments described above failed to show that nitrous acid has, in proportional to its probable ionisation, a more poisonous action that lactic acid, and the experiments with formaldehyde described below likewise fail to support

Loew's hypothesis, though the action of the poison is rather a remarkable one.

The eight experimental results obtained have been arbitrarily divided into two groups, according to the effects observed. Those reproduced in Fig. 18 show that the CO_2 output of the kidney was at a minimum immediately after poisoning with formaldehyde, and

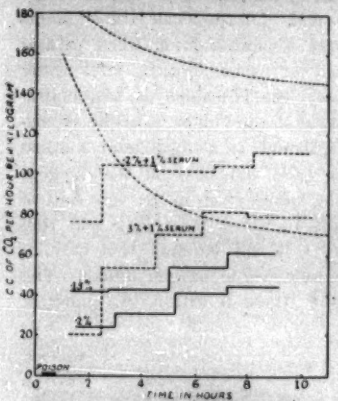

Fig. 18. Formaldehyde.

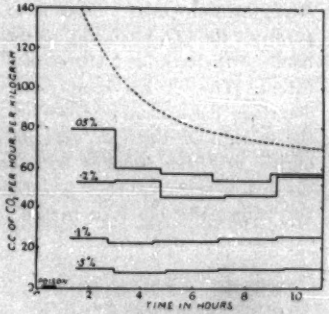

Fig. 19. Formaldehyde.

gradually rose to a maximum six to nine hours after the beginning of the experiment. The greatest effect was observed when a kidney was poisoned with saline containing ·3 % formaldehyde + 1 % serum, and was subsequently perfused with saline containing 1 % of serum. The CO_2 output then rose from 20 c.c. per hour in the 1½—2½ hour perfusion period up to 81 c.c. in the 6½—8 hour period. In the other experiment carried out in the presence of 1 % of serum, the kidney was poisoned with ·2 % of formaldehyde, and the CO_2 output increased from 76 c.c. per hour up to a maximum of 111 c.c. per hour in the 8½—10½ hour period. The two kidneys perfused with Ringer's solution showed a rise of CO_2 output from 42 to 61 c.c. and from 24 to 44 c.c. respectively.

In the four experiments represented in Fig. 19 the kidney was always perfused with saline containing no serum, and it will be seen that in one case the CO_2 output gradually diminished during perfusion, whilst in the three other cases it remained practically constant. However, this difference of result from the other set of experiments is not

so striking as it seems at first sight, for it does not apply, or only to a much more limited extent, to the oxygen intake values. In the table are given the respiratory quotients observed for the successive perfusion periods indicated in the curves in Figs. 18 and 19, and it will be seen that in every experiment except that in which ·05 % formaldehyde was used for poisoning, the quotient is low at first, and gradually rises throughout the course of perfusion till by the time of the last perfusion period it reaches more or less the normal. On the whole, the quotients in the earlier perfusion periods were somewhat lower when greater strengths of formaldehyde were used for poisoning than when lesser strengths were used. That is to say, the formaldehyde temporarily paralyses the CO_2-producing mechanism of the kidney as in the experiments with lactic and nitrous acids, though to a much more marked extent. That it has comparatively little influence on the oxygen-absorbing mechanism is shown by the values given in the right half of the table. Of the four experiments in which the CO_2 output rose during perfusion (marked with an *), it will be seen that only in the last of them was there a considerable rise of oxygen intake. The very slight rise observed in the other three is within the limits of experimental error.

Concentration of formaldehyde	Mean temp. of perfusion	Respiratory quotients	Oxygen intake in c.c. per hour per kilogram	Mean CO_2 output per hour, in c.c.
·05 %	16·9°	·70, ·75, ·80, ·79, ·80	112, 80, 70, 68, 72	60·4
·1	16·3	·57, ·63, ·64, ·76, ·82	42, 35, 36, 32, 30	23·6
*·15	16·3	·58, ·66, ·74, ·82	72, 65, 72, 74	49·4
·2	18·0	·48, ·62, ·65, ·66, ·75	111, 86, 70, 69, 79	51·0
*·2	16·6	·47, ·60, ·79, ·78	51, 51, 53, 57	37·3
*·2 +1 % serum	16·4	·65, ·75, ·83, .85, ·95	117, 139, 117, 123, 117	100·8
*·3 +1 % serum	16·8	·38, ·57, ·76, ·87, ·90	49, 87, 90, 93, 87	64·6
·5	16·1	·38, ·48, ·56, ·67, ·87	26, 17, 16, 14, 12	9·3

In the last column of the table is given the average output of CO_2 per hour during the 1½—11 hour perfusion period. Though the effects of poisoning with ·1 to ·2 % formaldehyde are rather irregular, it is evident that on the whole the greater the concentration of the poison the more was the gaseous metabolism depressed. Ignoring for the moment the two experiments carried out in the presence of 1 % of serum, the CO_2 output was highest in the kidney poisoned with ·05 % formaldehyde, and lowest in that poisoned with ·5 %. Of the two serum experiments, the kidney perfused with ·3 % formaldehyde had a considerably smaller CO_2 output than that poisoned with ·2 % formaldehyde.

The respiratory quotients.

As explained in previous papers, the estimation of the oxygen intake of a kidney is not so accurate as that of the CO_2 output, because any excess of oxygen in the saline after perfusion, over and above the ·7 c.c. per cent. which it contains when in diffusion equilibrium with the oxygen of the atmosphere, gradually diffuses into the layer of petroleum covering it, and so into the air; or conversely, if it contains less than ·7 c.c. per cent., oxygen diffuses into it from the petroleum. As far as possible, the kidneys were perfused with oxygenated saline at such a rate that the outflowing saline should contain more than ·7 c.c. of oxygen per cent. rather than less, so as to run no risk of oxygen want on the part of the kidney tissues. It generally contained about 1·10 c.c. or less, but even such an excess as this caused an appreciable error. The average errors arising with different amounts of oxygen excess were calculated from a large number of control observations, and have been used to correct the quotients given in the table below. In the quotients given in italics the correction amounted to more than ·10, and hence these values are not so reliable as the others, but even in their case the correction very seldom exceeded ·20.

Except when the quotients have already been recorded, they are given in the table in the order in which the experiments have been described. From the second column of the table it will be seen that the average temperature of perfusion did not vary very much. In the majority of the experiments the vessel containing the kidney and the perfused saline was placed in a large beaker of water, the temperature of which was kept slightly above that of the room by the radiation from a glow lamp placed 4 to 20 inches off.

Kidney poisoned with	Mean temp. of perfusion	Respiratory quotients
·025 % ammonia	15·4°	·89, ·81, ·76, ·87, ·68
·05 ,,	15·3	·82, ·75, ·80, ·82
·10 ,,	16·7	·59, ·63, ·67, ·68, ·56
·10 ,, +1 % serum	16·8	·70, ·69, ·76, ·77, ·89
·025 % methylamine	16·5	·83, ·72, ·74, ·71, ·65
·05 ,,	16·9	*·69*, ·66, *·74*, ·78, ·67
·10 ,,	16·2	*·42*, *·51*, *·49*, *·66*, ·77
·001 % mercuric chloride	17·0	·77, ·80, ·79, ·76
·01 ,,	17·8	*·92*, *·93*, *·95*, *·91*, ·81
·03 ,,	16·4	·80, ·72, ·71, ·72
·03 ,,	16·3	*1·24*, *·74*, *·68*, *1·05*, *·72*

Kidney poisoned with	Mean temp. of perfusion	Respiratory quotients
·10 % mercuric chloride	16·3	*1·10*, ·70, ·65, ·74, ·61
·10 ,,	16·4	·78, ·76, ·70, ·77
·40 ,,	16·5	1·17, ·80, ·73, ·68
·002 ,, throughout	17·2	1·18, ·75, ·83, ·82, ·80
·01 ,, ,,	16·9	1·00, ·76, ·93, ·84, ·84
·03 ,, ,, +1 % serum	16·2	·63, ·98, *1·04*, *1·13*, *1·12*
·05 % acid sodium sulphite	15·3	(1·10), ·72, ·76, ·71
·10 ,, ,,	14·7	(1·29), ·90, ·84, ·80
·15 ,, ,,	16·5	(*1·56*), ·89, ·62, ·68
·20 ,, ,,	14·4	(*3·00*), *1·16*, ·68
·20 ,, ,,	16·5	(*1·05*), ·63, ·88, ·98, ·94
·20 ,, ,,	18·3	(*1·85*), 1·13, ·88, ·81, ·79
·20 ,, ,,	16·7	(1·07), ·80, ·81, ·83, ·77
·25 ,, ,, +1 % serum	16·8	·81, ·80, ·87, ·83, ·84
·25 ,, ,,	18·5	(*2·60*), –, ·70, .80, ·87
·20 ,, ,,	16·5	(*1·14*), –, –, ·68, ·82
·1 % sodium fluoride + 1 % serum	16·0	*·68*, *·71*, *·95*, *·80*, ·77
·1 ,, ,, – $CaCl_2$	16·8	·65, ·74, ·89, ·80, ·76
1 ,, ,,	17·7	·70. ·80, ·83, ·80, ·78
1 ,, ,, +1 % serum	16·0	*·95*, *·98*, *·93*, ·87, ·84
1·5 ,, ,,	17·3	*·52*, *·82*, ·76, ·78, ·75
·5 % sodium oxalate + 1 % serum	16·2	·86, ·89, ·83, ·85, ·87
Saline – $CaCl_2$ throughout	16·4	·99, ·87, ·83, *·86*, *·87*, ·89
·1 % hydrazine hydrate	17·8	·82, ·87, ·81, ·80, ·75
·2 ,, ,,	17·4	·78, ·78, ·76, ·72, ·76
·05 % hydroxylamine	17·0	·90, ·95, ·78, ·81, ·72
·10 ,,	17·0	·95, ·88, ·80, ·75, ·78
·025 ,, throughout	14·5	1·32, 1·26, 1·11, 1·25
·10 ,, hydrochloride throughout	14·5	1·38, 1·06, ·78, ·71
·1 % phenyl hydrazine	16·5	·78, ·92, ·84, ·90
·15 ,, ,,	16·8	·85, ·96, ·95, ·89, ·91
·30 ,, ,,	18·8	·87, ·90, ·92, ·99
2 % urea	18·0	·93, ·95, ·94, ·91
2·5 ,, +1 % serum	16·8	·89, ·94, *1·00*, ·95, *·95*
3 ,,	16·8	·83, ·81, ·82, ·79, ·88
3 ,,	19·2	1·15, ·98, ·99, ·93
3 ,,	16·9	·86, ·79, ·80, *·80*
3 ,,	16·8	1·10, 1·13, 1·11, 1·11, 1·17
4 ,,	18·3	·87, ·85, ·83, *·87*, ·87
·20 % phenol + 1 % serum	16·8	·90, ·85, ·85, ·85, ·83
·30 ,,	16·9	·80, *·80*, ·79, ·82, ·75
·50 ,,	16·5	*·87*, *·88*, *·88*, *1·02*, *1·20*
1 ,, +1 % serum	17·0	*·76*, ·70, ·66
Saline ¼ sat. with chloroform	17·6	·82, ·82, ·80, ·80
,, ½ ,, ,,	17·0	*1·10*, *1·18*, *·82*, *·80*
,, sat. ,,	16·4	*1·23*, *1·28*, *1·05*, *1·00*

Kidneys poisoned with ·025 and ·05 % of ammonia gave normal quotients, but that poisoned with ·1 % gave distinctly low values, and that with ·1 % ammonia + 1 % serum, slightly low values. In a previous paper I showed that on continuous perfusion of kidneys with saline containing ·005 to ·025 % of ammonia, the quotients gradually sank to ·36—·49, and the present experiments indicate that a much shorter exposure to a greater strength of ammonia has a similar though less marked action on the CO_2-producing mechanism of the kidney tissues. This effect seemed to be permanent in one of the two experiments, but not permanent in the other. Probably the apparent permanence is not a genuine one, for not only was recovery of CO_2-producing power observed in kidneys poisoned with lactic and nitrous acids, and with formaldehyde, but the kidney poisoned with ·1 % of methylamine showed a very marked rise of quotient during the progress of perfusion. It began at ·42 in the first perfusion period, and increased gradually to ·77 in the last period. Another of the methylamine experiments showed a slight but apparently permanent lowering of quotient.

Poisoning with mercuric chloride probably has no effect whatever upon the quotient. It is true that the values given in the table are rather irregular in some experiments, but this is chiefly due to the fact that in their case the gaseous metabolism was small, and so the errors of experiment became magnified. Poisoning with acid sodium sulphite likewise seems to be without effect upon the quotient. The values obtained during the first perfusion period in these experiments are given in brackets, as they are vitiated by the presence of acid sulphite in the perfusion saline.

Poisoning with sodium fluoride seemed to depress the quotient for the first two hours or so of the subsequent perfusion period, and occasionally for longer, but the effect is never marked except in the experiment with 1·5 % NaF, and then only the first quotient obtained is low.

Probably neither hydroxylamine, hydrazine hydrate nor phenylhydrazine directly influences the quotient. As already mentioned, the high quotients obtained on continuous perfusion with saline containing ·025 % of hydroxylamine, are due to the spontaneous decomposition of this body. The two high values obtained in the hydroxylamine hydrochloride experiment are probably due to the same cause.

As might be expected, treatment of a kidney with 2 or 3 % urea solution has no effect upon the quotient, though in the experiment in which the CO_2 output of the kidney remained abnormally high

throughout the perfusion the quotient was likewise very high, *i.e.* 1·10 to 1·17. Phenol had no effect upon the quotient except when 1 % was used, and then it was apparently depressed a little. Poisoning with chloroform seemed to provoke a higher quotient, but it is possible that this result is a misleading one, and was due to the presence of traces of chloroform vapour passing from the kidney into the perfusion saline. Owing to its high tension, this would have the effect of increasing the apparent CO_2 output.

General conclusions.

From the mass of experimental data described in this paper it seems to me that two deductions may be made, which help us materially in understanding the mechanism of tissue respiration. The first deduction is that three substances, viz. hydrocyanic acid, acid sodium sulphite and sodium fluoride, can all enter into definite chemical combination with some constituent of the tissues, and thereby temporarily prevent these tissues from absorbing oxygen and eliminating carbon dioxide. This temporary union can be dissolved under suitable conditions, and the tissues then resume their original respiratory powers. Though there is no proof that these poisons exert their paralytic action upon respiration in the same way, by uniting to the same constituent groupings, the probability is that such is the case, and that in accordance with Loew's hypothesis they all of them unite with aldehyde groupings. These aldehyde groupings are of essential import in the respiratory process, and so long as they remain combined with any of the poisons mentioned, or with other substances, they are unable to function.

The second deduction concerns the CO_2-producing mechanism of the tissues. In the first place it is to be clearly borne in mind that this mechanism is distinct from the oxygen-absorbing mechanism. This was proved long ago by the experiments of Spallanzani, Pflüger[1], Aubert[2] and others on the capacity of frogs and other animals to continue evolving CO_2 for a long time after total deprivation from oxygen. This mechanism can be temporarily depressed by the action of poisons just as can the oxygen-absorbing mechanism, but probably in a very different way. Thus it is slightly injured by exposure for half an hour to the action of dilute acids and alkalis, but to get a marked

[1] Pflüger. *Pflüger's Arch.* VI. p. 43. 1871; X. p. 251. 1875; XIV. p. 5. 1878.

[2] Aubert. *Pflüger's Arch.* XXVI. p. 293. 1881. See also Vernon, *Science Progress*, II. p. 160. 1907.

effect it is necessary to let them act for some hours. Formaldehyde, on the other hand, has a marked effect even in half an hour. Now if acids and alkalis and formaldehyde all had a specific action upon certain constituent groupings of the tissues in the same way as HCN, NaF and $NaHSO_3$, they would presumably produce a definite effect in a comparatively short time, and this effect would not vary much over a considerable range of concentration of the poisons. But this is not the case. Acids and alkalis injure the CO_2-producing mechanism in proportion to their ionisation, and to the time for which they are allowed to act. The injury they produce is temporary, but it may take some hours to recover from, whilst the temporary depression of oxygen-absorbing power produced by the action of the poisons mentioned may under suitable conditions be recovered from much more rapidly.

Very suggestive work bearing upon the possible or probable mechanism of oxidation occurring in the tissues has recently been carried out by Dakin[1]. Starting from the discovery by Fenton[2] in 1894 that in the presence of a trace of a catalyst such as ferrous sulphate, hydrogen peroxide can readily oxidise tartaric acid and other organic substances, Dakin has extended the method to amino acids. He finds that bodies of the general formula $R.CHNH_2.COOH$ (*e.g.* leucin, alanin) are oxidised to CO_2, NH_3 and an aldehyde R.CHO. This aldehyde is then more or less completely oxidised to the corresponding acid, and this acid can then undergo further oxidation, and may in some cases, after passing through similar intermediate stages, be converted entirely into CO_2 and water. Similarly in the tissues it is possible that some chemical grouping may combine with oxygen from external sources to form a body of the nature of an organic peroxide, and then with the help of an intracellular peroxidase to serve as activator may transfer this oxygen to an acid molecule, and convert it into CO_2 and an aldehyde. As long as the aldehyde grouping remains free, it is capable of further oxidation, but if saturated by a molecule of HCN, $NaHSO_3$, NaF or other substance, then for the time being the oxidation process must come to an end.

Carbohydrate molecules would be oxidised in the tissues in the same way as acid molecules, by having their terminal CHO or CH_2OH grouping oxidised first to COOH, and then split off as CO_2. Similarly also the annexation of an HCN or other molecule by the CHO grouping would for the time being prevent further oxidation.

[1] Dakin. *Journ. Biol. Chem.* IV. pp. 63, 77, 81, and 227. 1908.

[2] Fenton. *Journ. Chem. Soc. Trans.* p. 899. 1894.

In attempting to explain the action of acids, alkalis and formaldehyde upon the CO_2-producing mechanism of the tissues one has to bear in mind that these poisons do not stop oxidation altogether. They simply stop the oxidation from proceeding as far as the final CO_2 stage. Apparently, therefore, there are two grades of oxidation occurring in the tissues, only the more vigorous and complete of which is temporarily injured by formaldehyde. Arguing from the above quoted experiments of Fenton upon the oxidising power of hydrogen peroxide, which is enormously increased by the presence of the trace of an activator, it is possible that the action of formaldehyde, acids and alkalis is chiefly upon the sensitive peroxidase activator of the tissues, whilst it leaves the organic peroxide comparatively unharmed. This organic peroxide can still absorb oxygen from its surroundings, and transfer it to easily oxidisable substances in the tissues, but it is only when it is assisted by a peroxidase that it can oxidise them completely to CO_2 and water. The gradual recovery of CO_2-producing power by the poisoned tissues is presumably due to the gradual regeneration of fresh peroxidase.

The fact that oxidation can occur in the tissues without the immediate production of CO_2, is suggested by certain of Fletcher's experiments upon the respiration of excised frogs' muscles. Fletcher[1] observed that tetanisation of gastrocnemii kept in air or nitrogen caused little if any increase of CO_2 production, but if they were kept in oxygen, the CO_2 output was more than doubled. In the absence of sufficient oxygen, the oxidation of the extra products formed in the tissues by the activity of the muscle stopped short of the final CO_2 stage. Fletcher did not estimate the respiratory quotient of the muscles, but there is no reason for thinking that it was specially influenced by the condition of activity or of rest. Hence the incomplete oxidation observed by him is not comparable to that produced in the kidney by poisoning with formaldehyde. It simply shows that when the supply of oxygen is limited the oxidation processes do not occur so vigorously as when it is unlimited.

It will be remembered that the gradual deterioration of the respiratory power of the tissues produced by perfusion with Ringer's solution, or by other abnormal conditions, does not influence the respiratory quotient. Hence one might argue that it affects the oxygen-absorbing and CO_2-producing mechanisms equally. But considering how differently these mechanisms are constituted this is not likely to be the case, and indeed the assumption is quite unnecessary. If the first

[1] Fletcher. This *Journal*, XXIII. p. 10. 1898; XXVIII. pp. 354 and 474. 1902.

step in the oxidising process is prevented, then the whole chain of events inevitably collapses. That is to say, the process is entirely dependent on the presence in the tissues of a chemical grouping which can loosely bind oxygen derived from external sources, and if this is wanting, respiration must cease. The extremely small oxidising capacity possessed by animal tissues after disintegration is probably due to their intracellular peroxide being rapidly destroyed by acids and other disintegration products.

This hypothesis, however, I regard as purely provisional.

Summary.

The action of various poisons upon tissue respiration was investigated by perfusing freshly excised rabbits' kidneys for half an hour with saline containing the poison, then perfusing continuously with oxygenated saline for 10 hours, and estimating the CO_2 produced and oxygen absorbed.

Poisoning with ·025 to ·1 % of ammonia and of methylamine reduced the CO_2 output to $\frac{1}{2}$—$\frac{1}{3}$th the normal, the effect produced being proportional to the concentration of the alkali. Hydrazine hydrate and hydroxylamine in ·05 to ·20 % solution had a smaller effect, or the toxic action of these four alkalis was in rough proportion to their ionisation.

Lactic acid in ·2 to ·4 % strength had about as much effect as ·02 to ·03 % of nitrous acid (containing nitric acid), or again the effect was proportional to the ionisation.

Poisoning with any concentration of mercuric chloride from ·01 to ·4 % produced almost the same effect, the CO_2 output being reduced to a half or third the normal.

Kidneys when poisoned with ·05 to ·25 % of acid sodium sulphite, and then perfused with saline containing no $NaHCO_3$, had a permanently low gaseous metabolism. But if saline containing ·02 or ·03 % of $NaHCO_3$ were used, the metabolism rapidly increased till after 6 or 8 hours it became doubled in amount and attained the value shown by normal unpoisoned kidneys. Even if a kidney poisoned with $NaHSO_3$ were perfused with alkali-less saline for 4½ hours, its respiration at once shot up on substituting saline containing $NaHCO_3$. Hence it is thought that the acid sulphite was in loose combination with some chemical groupings (probably aldehyde) in the tissues, whereby their respiration was paralysed, and that the $NaHCO_3$ set it free and so restored the respiratory power.

Poisoning with sodium fluoride also yielded evidence of loose combination with the tissues. Thus almost exactly the same effect was produced by 1 % NaF as by ·1 %, and the presence or absence of serum or of calcium salts made no difference. The CO_2 output was always low at first (43 to 52 c.c. per hour), and then rose in the next three hours or so to a maximum of 77 to 94 c.c. per hour, or the same value as that of normal unpoisoned kidneys. Fluorides are known to be capable of forming combinations with aldehydes, but so are hydroxylamine, hydrazine hydrate, phenyl-hydrazine and urea, and no evidence was obtained in their case of the formation of loose combinations with the tissues, and temporary paralysis of respiration. However, hydrocyanic acid has been shown to form such a combination.

Poisoning with ·2 to 1 % of phenol depressed the respiratory powers of the tissues in proportion to the concentration. Probably a similar relation holds for chloroform of various concentrations.

Formaldehyde (·05 to ·5 %) probably injures the tissues in proportion to its concentration, but it temporarily paralyses the CO_2-forming mechanism much more powerfully than the oxygen-absorbing mechanism. Hence in the two hours after the poisoning the CO_2 output is low and respiratory quotients of ·38 to ·58 are obtained. During the next four hours or so the CO_2 output and the quotients gradually rise till they may ultimately attain the values of normal unpoisoned kidneys. Lactic and nitrous acids have an analogous though less marked effect, whilst it has been shown in a previous paper that continuous perfusion with saline containing ·005 to ·025 % of ammonia, or ·1 to ·2 % of lactic acid, gradually lowers the quotient to ·36—·46. Other poisons do not affect the quotient.

The following hypothesis of the mechanism of tissue respiration is suggested.—The tissues contain a substance which can absorb oxygen from their surroundings to form an organic peroxide, and by the help of a peroxidase can transfer this oxygen to amino acids and carbohydrate molecules bound up in the tissues. The reaction is similar to that of H_2O_2, which in the presence of an activator, can oxidise acids of the formula $R.CH.NH_2.COOH$ to CO_2, NH_3 and an aldehyde $R.CHO$, and then oxidise this aldehyde to $R.COOH$ and ultimately to CO_2 and H_2O (Dakin). Poisons such as HCN, $NaHSO_3$ and NaF temporarily paralyse respiration by uniting with the aldehyde groupings. Poisons such as formaldehyde, acids and alkalis temporarily paralyse the CO_2-forming power of the tissues by destroying the peroxidase. The organic peroxide, though it can still effect some oxidation, cannot

of itself carry it to the final CO_2 stage. Recovery of CO_2-forming power is due to regeneration of peroxidase. The slow deterioration of respiratory power produced by prolonged perfusion with saline and by other abnormal conditions, is due primarily to destruction of organic peroxide.

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THE INFLUENCE OF THE VISCERAL NERVES UPON THE HEART IN CEPHALOPODS.

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THE object of this research was to re-investigate the action of the visceral nerves upon the heart in Cephalopods, since the results obtained by previous observers are not wholly in accord, especially with reference to the presence of augmentor (accelerator) fibres. The position occupied by the cephalopod molluscs as the highest of the Invertebrata, the remarkable degree of development of their circulatory apparatus and its similarity in many respects to that of Vertebrata, lend a general interest, from the point of view of the comparative physiology of the vertebrate and invertebrate heart, to a study of the mode of action of their cardiac nerves.

The influence of the visceral nerves upon the heart in Cephalopods was first discovered in Sepia in 1867 by Paul Bert[1] who described their inhibitory function on stimulation with an induction current, a result confirmed by all subsequent observers with the exception of Dew-Smith[2] in 1874, who could discover no inhibitory nerve in either Sepia or Aplysia. In 1878 Fredericq[3] stated the presence also of accelerator nerves to the systemic heart of *Octopus vulgaris*, but Ransom[4] in 1884 denied the existence of such fibres either bound up in the visceral nerves or running as separate nerves. Fuchs[5] in 1895 ascribed to them in addition a vaso-motor function. Bottazzi and Enriques[6] in 1900 stated the presence of separate augmentor nerves running from the gastric ganglion; but Carlson[7] in 1905 was unable to confirm this statement or that of Fuchs', and considers that the visceral nerves probably also contain augmentor fibres to the ventricle, though he admits the evidence to be inconclusive.

Preparation. Except where specially mentioned *Eledone moschata* was the subject of experiment, the anatomy of which is given by Fuchs[8],

and of Octopus by Ransom[4]. In order to prevent errors due to the contraction of other muscular structures supplied by the visceral nerves, the heart was excised for registration. The animal having been secured for dissection according to the method employed by Fuchs[5], the mantle cavacity was laid open from the (physiologically) ventral surface, the right and left visceral nerves were dissected out in their full length and severed close to the visceral ganglion. Having tied the vena cava, the ventricle was laid bare by removal of the renal veins and their appendages. After ligation of the abdominal aorta, the genital artery, and the auricles close to the gills, these were then severed. A small graduated glass cannula was tied into the commencement of the cephalic aorta. After severing the latter and other attachments, the ventricle and the auricles with the visceral nerves could then be removed. The heart-beat rapidly ceased on exposure to the air and consequently all dissection had to be done under well oxygenated sea water. After excision the heart was suspended vertically by means of the cannula in a fluid through which oxygen was bubbled during the course of the experiment. The cannula was also filled with the same fluid, by means of which the pressure in the heart was raised to the point at which the maximal heart-beat was obtained.

Recording arrangements. For graphic registration of the ventricular beats a small hook, connected with a light lever by means of a thread working round a pulley, was inserted into the ventricular wall close to the junction of the abdominal aorta with the ventricle, as in the method used by Ransom[4]. The auricular contractions were recorded, either by inserting the cannula into the cut gill end of the auricle and freeing the auricle from the ventricle close to the auriculo-ventricular junction, leaving a small portion of the wall of the ventricle for the insertion of the hook attached to the lever, or using the above preparation with the auricle in connection with the ventricle, by fastening the cut gill end of the auricle to the thread attached to the lever, at the same time making fast the ventricle to a cork plate in the damp chamber in such a way as to prevent the registration of the ventricular beats. Owing to the peristaltic nature of the auricular contractions the curves registered were of an irregular nature.

For simultaneous registration of the beats of the auricles and ventricle, a specially constructed damp chamber was used, consisting of an ebonite chamber with removable glass walls at the front and back. To the side walls were attached adjustable pulleys for the threads from the auricles. To the floor was fixed a pulley for the thread from the

ventricle and a tube for the oxygen supply. The heart cannula was held vertically by a clip on the posterior wall, and the platinum electrodes for the nerves were similarly adjustable on the walls. The chamber was half filled with saline fluid and the heart immersed in it. The threads from the two auricles and the ventricle were attached to three straw levers constructed to write one below the other, above the levers of an electric signal and a Jacquet time marker. The complication of technique involved in the use of five levers simultaneously was necessary in some experiments in order to obtain decisive results. Four Daniell cells were usually employed. All curves are to be read from left to right, upstroke = contraction. Time is given in seconds. The downstroke of the electric signal during stimulation indicates a make shock. Except where specially mentioned, the upper curve of the heart tracings is of the left auricle, the middle of the ventricle, and the lower of the right auricle.

General factors influencing the rhythm of the excised heart.

Since in any excised muscle-nerve preparation the functional capacity, not only of the muscle itself but of the nerve fibres and nerve endings in indirect stimulation, is determined by the character of the muscular contractions, it is necessary to consider the general conditions affecting the contractions, and this is of special importance when the muscle is in a condition of rhythmical activity. The conditions investigated were: the nature of the saline medium, the effect of oxygen, the pressure of fluid within the heart and the temperature.

Saline medium. Sea water (Ransom[4]) was the fluid first used, both within and without the heart, but owing to the fact that the heart-beat was not always quite satisfactorily maintained for a long period, experiments were made with a modified Ringer's solution of the following formula, suggested by Dr Führer:—$NaHCO_3$ ·1 gm., $CaCl_2$ (dry) ·2 gms., KCl·1 gm., NaCl 34·5 gms., Aq. dist. 1 litre. With pure sea water the rhythm of the heart remained quite regular, but the beats averaged only about 20 per minute, instead of 35—38 per minute as in the normal animal (Fuchs[5]). On replacing the sea water in the cannula by the Ringer's solution, the rhythm immediately became very rapid, about 45—48 beats per minute, with a rise of tonus at intervals. On further replacing the sea water outside the heart by Ringer's solution the rhythm became slightly more rapid, about

50—55 per minute, with more frequent rises of tonus. On replacing the pure Ringer solution, within and without the heart, with a mixture of $\frac{1}{3}$ Ringer and $\frac{1}{3}$ sea water the rhythm at once became more regular and the beats were reduced to 18—20 per minute, while the size of the beats was somewhat increased. A mixture of $\frac{1}{4}$ Ringer and $\frac{3}{4}$ sea water caused no alteration in the rhythm. The Ringer's solution has thus a direct stimulating action upon the heart, and this was also shown by the fact that Ringer's solution introduced into a previously quiescent heart always set the heart beating at a rapid rhythm. After cessation of the beat in Ringer's solution, transference to pure sea water roused the beat again so that sea water had a directly antagonistic action to the Ringer's solution, but the resultant effect of equal parts of the two solutions was not a mean of their separate effects. The fact, however, that with certain specimens no adjustment of the proportions would maintain the rhythm at all, while in other cases, with either pure sea water or a solution of $\frac{1}{2}$ Ringer, $\frac{1}{2}$ sea water, the rhythm remained quite regular for many hours, suggested that in the former case the result was due rather to some unknown factor in the nutrition of the specimen than to an incorrect molecular concentration of the fluids used or to the absence or incorrect proportion of some particular constituent. The solution $\frac{1}{3}$ Ringer and $\frac{1}{3}$ sea water was generally employed. Though not universally satisfactory, yet the conditions obtained by its use were not such as to affect the validity of the subsequent experiments.

Oxygen. Fuchs[m], Bottazzi[n] and Carlson[o] have noted the quick cessation of the heart-beat on exposure to the air, and it was found that the heart was very sensitive to the presence of O_2, since a previously quiescent heart frequently commenced to beat spontaneously directly after the introduction of oxygen into the surrounding fluid; while the heart-beat rapidly failed in its absence. If Luciani groups appeared, the experiment was abandoned.

Pressure. The heart muscle was considerably influenced by tension, since a resting ventricle often commenced to beat spontaneously on increase or diminution of the pressure of the fluid within the heart. The pressure of fluid most favourable for the maintenance of the rhythm varied from about 2·0—6·0 c.c., but the average was about 4·5 c.c.

Temperature. Medium temperatures about 16·0° C. were found more favourable to the rhythm than high, the highest recorded temperature at which the heart continued to beat for any considerable length of time was 20·5° C. The importance of the temperature

appears probable from the fact that the ventricular beat could not be maintained in summer specimens even in water artificially cooled to 16° C., though the auricular beats continued. It cannot however be definitely said that the functional activity of the heart of such specimens is depressed by the continuous action of the high temperature of the water, 23°—25° C., rather than by some other unknown physiological factor.

Effect of indirect stimulation with the interrupted current.

A. *Beating ventricle.*

Strength of stimulus. The normal effect of stimulation of the left visceral nerve in males with currents of moderate strength (coil distance 100—150 mm.) is to cause inhibition of the ventricle, usually with some diminution of tonus (Fig. 1). If a contraction has once commenced it is always completed without any diminution of its height, at whatever period of the contraction the stimulus may have been introduced, but if the stimulus falls during or immediately after diastole,

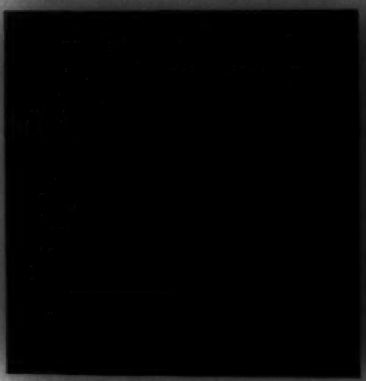

Fig. 1.

Fig. 2.

Fig. 1. × ⅓. Upper line, Rt. Auricle. Middle line, Ventricle. Lower line, Lt. Auricle. Coil distance 100 mm. Frequency 47 per sec. Contraction of left auricle checked by a stop.

Fig. 2. × ⅓. Upper line, Ventricle. Lower line, Lt. Auricle. C.D. 100 mm. Fr. 47 per sec.

then inhibition at once occurs. After cessation of the stimulus there is an increase in the height of contraction with an increase of tonus and usually, but not invariably, some quickening of the rhythm.

With strong stimuli the fall of tonus is greater, and if the stimulus coincides with a ventricular systole there is some diminution of the height of contraction with a strong rise of tonus after the cessation of the stimulus. Stimulation of the right nerve in males, even with the strongest currents, has, however, no effect at all. This was a quite constant result with all specimens of males examined, whatever strength or rate of stimulus was employed. In females, however, both nerves were functional in the normal manner, sometimes the right, sometimes the left being the more effective. The right nerve was always found to be decidedly thinner than the left in males, due perhaps to the asymmetry of the latter, and this may possibly account for the difference in action of the two nerves by a diminution of the number of fibres involved, as has been suggested in other cases.

Tarchanoff[m] obtained practically identical results to the above in both amphibia and mammals, finding that the period at which the vagus most quickly took effect was at the end of diastole and commencement of systole, but that a pulsation invariably occurred between the moment of stimulation and the moment of arrest, however strong the stimulus. He found, however, the right nerve to be more effective than the left in amphibia and mammals.

After a varying period, however, a progressive change in the effects of stimulation gradually occurs. With the same strength of stimulus as before, the inhibitory effect is at first still obtained, but on cessation of the stimulus the after effects are enormously increased, the contractions progressively increasing and the relaxations becoming diminished so that there is a "staircase" effect causing a very great rise of tonus (see Fig. 13). Still later, there is a slight inhibition followed by beats increasing in height during the passage of the stimulus with the same staircase effect after cessation of the stimulus, followed by a relaxation and sometimes cessation of the heart-beat for a short period (see Fig. 14). This is followed by a condition, in which, on stimulation with the same strength of current, instead of the normal inhibitory effect, quite the opposite is obtained, and an apparent tetanus of the ventricle occurs throughout the duration of the stimulus. If the stimulus is prolonged, beats occur both during and after cessation of the stimulus, the height of the beats varying inversely with the height of the tetanic effect (Fig. 2). The nature of these apparent tetanic effects has been discussed

by Bassin[38]. Ransom[40] obtained somewhat similar results to Fig. 14, attributing the increase in the height of the beats during the stimulus to an increased excitability of the ventricle set up by nerve stimulation with strong currents. This is not very probable since a very slight diminution of the strength of the stimulus may sometimes restore the inhibitory effect, and moreover the stimulus remains of exactly the same strength as that which previously caused inhibition, so that the motor effect is not due to the current being of too great strength. Carlson[7] also observed a similar "paradoxical" effect, which he considers to be evidence in favour of the presence of augmentor fibres in the visceral nerves.

Frequency. Unlike the case in mammals, inhibition was less dependent on the frequency than on the strength of stimulus, since the frequency of stimulation necessary to cause complete inhibition varied inversely with the strength of the stimulus. The lowest rate of stimulus found capable of causing complete inhibition, coil distance 100 mm., was 35 per minute, and the threshold values were sharply defined, since a rate of 27 per min. was quite ineffective with the same strength of stimulus.

Duration. A moderate strength of stimulus at a rate of 1 per second was found just adequate to cause complete inhibition. If this was maintained for a considerable period, the same phenomenon of beats, increasing in size during the stimulus, was observed. This result was evidently not due to a strength of stimulus beyond the inhibitory optimum (Ransom[40]), since a stimulus of the same strength, of greater frequency, but of short duration caused complete inhibition throughout the passage of the stimulus, and the after effect was correspondingly increased. Stimulation of the same visceral nerve can thus cause either an inhibitory or an augmentor effect in the systemic ventricle. The change in the action of the nerve upon stimulation might be explained by one of the following three suppositions.

1. By the simultaneous stimulation of two sets of fibres, augmentor and inhibitor, the resultant effect of which is the algebraic sum of their activities, the augmentor set finally gaining an ascendancy owing to the fatigue of the inhibitor, or,

2. By the anabolic theory of inhibition, supported by Ransom, or,

3. To some change in the neuro-muscular mechanism.

The first hypothesis appears to be negatived by the action of the nerves upon the resting ventricle.

B. *Resting ventricle.*

Immediately upon stimulation of the nerve there occurs a marked rise of tonus, which persists throughout the period of stimulation, occasionally with small fluctuations. On cessation of the stimulus the heart at once commences to contract regularly, usually with a diminishing rate and height of contraction. The degree of tonus rise increases with an increase in the strength of the stimulus. The right nerve in males has no effect on the resting ventricle (Fig. 3). If now, during the beats thus evoked, a stimulus of exactly the same strength and frequency is introduced, inhibition may at once ensue in the normal manner throughout the duration of the stimulus. (Fig. 4). This demonstrates that the motor effect of the nerve is not due to its becoming unmasked by the fatigue of the inhibitor fibres, nor to any stimulation of the ventricle by escape currents. Though the effect of stimulation of the nerve is evidently conditioned to some

Fig. 3. ×½. C.D. 100 mm. Fr. 47 per sec.
Fig. 4. ×½. C.D. 100 mm. Fr. 47 per sec.

extent by the state of the ventricle, the effect is not mainly dependent upon this factor, since the motor effect has already been observed also in the beating ventricle. Bottazzi and Enriques[6] and Ransom[4] have noticed the evocation of beats, though not the rise of tonus, on stimulating the nerve to the resting ventricle, but attributed it to the anabolism set up by the nerve stimulation. It remains therefore to examine the other two theories mentioned above, and to determine which is the more in accordance with the facts.

EFFECT OF SINGLE INDUCTION SHOCKS ON THE BEATING VENTRICLE.

Indirect stimulation. Single induction shocks were found capable of causing inhibition of the ventricle. The length of inhibition was affected by the strength and the nature of the stimulus. A make shock (coil distance 100 mm.) applied to the nerve at the commencement of an experiment, set up an inhibition lasting 10—12 seconds (Fig. 5); at later periods increased strengths of shock were necessary to cause the same length of inhibition. Within limits, increase in the strength of the shock caused an increase in the length of inhibition, with some rise of tonus at its commencement.

Strong shocks, coil distance 50—60 mm., caused inhibitions which frequently lasted 20—30 seconds.

Break shocks were found to be less effective than make in causing inhibition, break : make :: 80 : 100 mm., coil distance.

Fig. 5. $\times \frac{1}{3}$. C.D. 100 mm.

The height of the first contraction subsequent to inhibition was slightly increased, but the succeeding contractions, unless a strong shock was used, were normal and the rhythm was not increased. If the stimulus was of sufficient strength inhibition always ensued, whatever might be the period of the cardiac cycle with which the stimulus was coincident. If, however, a contraction had once commenced, even if the stimulus fell at the very commencement of systole, the latter was completed without any diminution in the height of the contraction which was then followed by the period of inhibition, while if the stimulus fell during diastole or in the period before the commencement of systole inhibition at once ensued, a result exactly similar to that obtained with the interrupted current (Fig. 5).

It is of interest to contrast these effects obtained with single induction shocks upon the visceral nerves (vagus) of cephalopods with those upon the vagus of mammals and amphibia. There is apparently no other instance recorded in the literature of the cardiac nerves of a single induction shock being a stimulus of sufficient duration to cause

inhibition. Tarchanoff[9] indeed found it quite impossible to obtain any arrest of the heart in mammals or amphibia with single induction shocks however strong (large Ruhmkorff coil and 6 Bunsen cells), several successive induction shocks being always required. The vagus in mammals is therefore more dependent for its action upon the duration than upon the intensity of a stimulus, while the former factor is much less important in the case of the visceral nerves, which do not require a summation of stimuli in order to set up inhibition. In this connexion it is interesting that Legros and Onimûs[10] found that a more rapid rate of interrupted current was needed to cause inhibition in warm-blooded than in cold-blood animals.

These results with single induction shocks may be regarded as the corollary of those mentioned above with low frequencies of the interrupted current.

Direct stimulation. A direct stimulus with a single induction shock caused an extra systole if it did not fall during the refractory period, though Carlson[9] indeed has shown that in invertebrates and many vertebrates the refractory period is only one of reduced excitability and not of complete inexcitability. It was found that the compensatory pause and "bigeminus" (Hering[10]) was full when the extra systole fell at the end of the previous diastole, and was shortened if it fell at the commencement, as was found to be the case with the mammalian heart by Hirschfelder and Eyster[11].

Fig. 6. × ½. At A. Indirect stimulus. Single ind. shock C.D. 50 mm.
At B. Direct " " " " C.D. 50 mm., causing an extra systole. Inhibition lasting 45 secs.

The nature of the inhibition caused by the stimulation of the visceral nerves was now investigated.

If, during the inhibition set up by a single induction shock applied to the nerve, a direct stimulus was applied to the heart muscle by means of a single induction shock of the same or slightly greater

strength immediately after the stimulus causing inhibition, it was found that an extra systole could be evoked. This extra systole was then followed by an inhibition, the duration of which was dependent on the above mentioned factors influencing inhibition. With a strong stimulus on one occasion it lasted up to 45 seconds (Fig. 6). The time interval between the two stimuli had no effect upon this extra systole, which could be just as readily evoked immediately after the inhibitory stimulus as at a later period, nor was it affected by the period of the cardiac cycle in which the inhibitory stimulus was incident, thus, if the inhibitory stimulus fell during systole, the extra systole could occur during the following diastole (cf. Fig. 6), while if the inhibitory stimulus fell during or after diastole, the extra systole occurred during the inhibition thus set up.

Exactly the same strength of current evoked an extra systole and its height remained the same during an inhibition as when an inhibition had not been set up.

Therefore an inhibition set up by stimulation of the visceral nerves caused no diminution of excitability or functional capacity in the heart muscle. In view of this result it seems possible that the inexcitability of the mammalian heart muscle to direct stimulation during vagus inhibition may be due to the necessity of employing an interrupted current.

This experiment threw some light on the nature of the inhibition and was opposed to Ransom's[40] explanation of the inhibition as caused by anabolic processes set up by the nerve suppressing the catabolic processes in the muscle, since on that hypothesis the heart muscle should have been inexcitable to a direct stimulus. On the other hand it pointed directly to the fact that the inhibitory process was situated in the nerve or its terminal apparatus and not at all in the muscle itself, and showed that the inhibition was not a prolongation of the refractory period of the muscle.

Since, however, it was possible that the ventricular inhibition might be due to a diminution of the normal excitation arriving at the ventricle from the auricles, consequent upon an inhibition of the auricular contractions, investigations were next made upon the influence of the visceral nerves upon the auricles. Here it may be mentioned that the auricle is composed mainly of non-striated muscle.

EFFECT OF INDIRECT STIMULATION ON THE BEATING AURICLE.

Both Ransom[6] and Carlson[7] state that inhibition of the auricle is the normal effect of stimulation of the visceral nerve on the same side. Not only was this not found to be the case in Eledone, but quite the opposite effect was normally obtained.

Single shocks. With a break shock of moderate intensity, coil distance 100—150 mm., there was immediately a sharp contraction. The height of contraction was affected by the conditions affecting inhibition in the ventricle, viz. the strength of the stimulus and its nature. The height of contraction was increased by an increase in the strength of the stimulus, while make shocks were usually almost ineffective in causing a contraction (Fig. 7).

Fig. 7. × ½ about.

The above results were therefore the exact converse of those obtained with the ventricle, but the progressive change in the response to nerve stimulation mentioned above in the case of the ventricle was not observed. There was no subsequent increase of tonus or rhythm or of the strength of the peristaltic contractions. The contraction could occur at any period of the peristalsis, whether it was at its maximum or its minimum (Fig. 7), so that there was apparently no refractory period. That these results were not due to escape currents stimulating the auricle was proved by the absence of any contraction in control experiments in which the nerve was destroyed by heat distal to the point of excitation.

Interrupted current. From the above results it was probable that the interrupted current of normal strength, coil distance 100—150 mm., would cause a tetanic contraction of the auricle, and this was found to be the case (Fig. 8 A). The contraction was immediate, large, and, at the

commencement of stimulation, very rapid, but gradually became slower during the passage of the stimulus, ceasing immediately on cessation of the stimulus, and was followed by a fall of tonus, but the diminution of the tonic condition was extremely gradual, so that the auricle remained within the limits of observation in a more or less contracted condition. Anatomical examination showed that this result was not due to any peri-auricular sheath masking the true response of the organ, such as Carlson[7] has shown to have been a source of error in the case of the branchial hearts. If now, during the contracted condition of the auricle, the wall of the ventricle was directly stimulated near the exit of the abdominal aorta with the same strength of current as applied to the visceral nerve, there was observed instead of a contraction a diminution of tonus in the auricle, and the fall of tonus increased with an increase in the strength of the stimulus. The normal effect of contraction of the auricle on stimulation of the visceral nerves was therefore not due to escape currents.

Fig. 8. $\times \frac{1}{3}$ about. Stimulus A. C.D. 150 mm. Fr. 47 per sec.
Stimulus B. C.D. 250 mm. Fr. 47 per sec.

If however the nerve was stimulated with feeble strengths of current, coil distance 200—250 mm., then, instead of the tetanic contraction, a diminution of tonus was observed, and the fall of tonus increased within limits with a decrease in the strength of the stimulus (Fig. 8 B). The results obtained with the auricle are, therefore, exactly the reverse of those obtained with the ventricle, the effect obtained being dependent on the strength of the stimulus. Stimuli, however, which cause inhibition of the ventricle, cause contraction of the auricle, so that the inhibition of the ventricle is not in any way caused by a diminution of the auricular contractions. Since it might be argued that these opposite effects were obtained in the isolated auricle and ventricle, confirmation of this result was necessary by the simultaneous registration of the

combined response of the auricles and ventricle to stimulation of the visceral nerves.

Simultaneous records of auricles and ventricle with indirect stimulation.

A. *Beating Heart.*

Interrupted current of short duration. With stimuli of normal intensity, coil distance 100 mm., inhibition of the ventricle takes place at once in an exactly similar manner to that mentioned above in the case of the isolated ventricle, while at the same time there is an immediate sharp contraction of the auricle of the same side as the nerve stimulated. Both the inhibition of the ventricle and the tonic contraction of the auricle are maintained throughout the duration of the stimulus. On cessation of the stimulus the inhibition of the ventricle and the tonic contraction of the auricle at once cease, and are followed by the usual increase in the height of the contractions and the rhythm of the ventricle and by a very gradual diminution of the auricular tonus, so that the tonic condition of the auricle

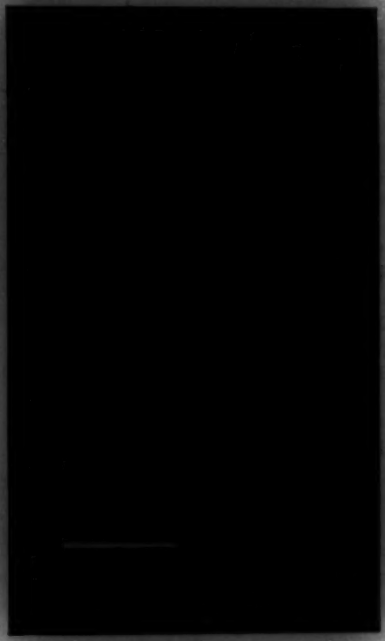

Fig. 9. × ½. Left nerve stimulated. C.D. 100 mm. Fr. 47.
Effect on right auricle due to traction.

lasts long after the ventricle has attained its normal rhythm (Fig. 9). The "physiological isolation" of the two organs is thus strikingly apparent.

Ransom[40] has indicated the anatomical basis of this fact by the almost complete interruption of continuity of the muscular fibres of the organs at the auriculo-ventricular junctions.

The inhibition of the ventricle is thus not caused by any diminution of a normal stimulus reaching it from the auricles, and it must therefore be concluded that the seat of inhibition of the ventricle is situated in the nerve or its terminal apparatus.

Fig. 10. ×½. Upper tracing, Rt. Auricle. Middle tracing, Ventricle. Lower tracing, Lt. Ventricle. Left nerve stimulated. C.D. 200 mm. Fr. 95 per sec.

The opposite effects given by the auricle and ventricle respectively may be explained either (1) by the visceral nerve containing two sets of fibres of opposite nature, or (2) by the same fibres setting up different reactions in each organ.

Stimuli of a strength insufficient to cause inhibition of the ventricle were found to evoke no contraction in the auricle, the threshold stimulus causing the one reaction being also the threshold stimulus for the other. If the stimuli are of sufficient strength to cause inhibition of the ventricle the height of the auricular contraction increases with an increase in the strength of the stimulus.

If, then, the visceral nerve contains motor fibres for the auricle and inhibitor for the ventricle, it must be supposed that the motor fibres have the same threshold stimulus as the inhibitor, which is not at all probable.

Further, if the nerve is stimulated with currents of very weak intensity, coil distance 250—300 mm., then instead of inhibition there occurs a slight increase in the contraction and relaxation of the ventricular beats, while at the same time there is a slight but quite definite

diminution of the auricular tonus in the place of contraction (Fig. 10). This change in the reaction of the auricle and ventricle to nerve stimulation by a change in the strength of the stimulus is somewhat comparable to the Wedensky effect[13] and is perhaps explained by the recent work of Keith Lucas[16] on optimal electrical stimuli. On the supposition that there are separate fibres for each function, there would have to be contained in the visceral nerve four separate sets of fibres, the fibres with opposite functions in the auricle and ventricle having similar stimulation values and *vice versa*. But this appears inherently improbable, and is unsupported by any direct evidence.

The above results can be more simply explained by supposing that the same nerve can conduct both inhibitory and excitatory impulses and that the nature of the reaction obtained is determined by the nature of the end organ or "receptive" substance in which the fibres terminate. Thus it has been shown above that the seat of inhibition in the case of the ventricle is probably not in the muscle itself, but in the nerve or its terminal apparatus, and it is probable therefore that the differences in reaction of the auricle and ventricle are not due to the actual differences in their muscular structure but to modifications of the terminal apparatus of the nerve caused by such structural differences (cf. Langley[14]). This would accord directly with the facts, since on such a hypothesis opposite reactions in the auricle and ventricle would naturally be obtained by stimulating with the same strength of current, the same nerve having different terminal apparatus in the two cases, and this would perhaps also account for the opposite reactions obtained with the beating and resting ventricle, since Piotrowsky[17] in his experiments on the antagonistic muscles of the crayfish claw, which bear a certain resemblance to these opposed reactions of the auricle and ventricle, found that the effect obtained on stimulation differed with the degree of muscular tonus.

Interrupted current of long duration. With stimuli of normal strength but of long duration, whilst the ventricle remains completely inhibited throughout the period of stimulation, the auricular effects vary. This variation is illustrated by the following two cases. In the one the auricular contraction falls during the period of stimulation almost to its original height (Fig. 11), in the other the original auricular contraction falls and there then occurs a second rise followed by a second fall during the period of stimulation (Fig 12). These two typical cases confirm in a different manner the above mentioned physiological independence of the two organs.

Fig. 11. × ½. Left nerve stimulated. C.D. 100 mm. Fr. 47.
Contraction of left auricle checked by stop.

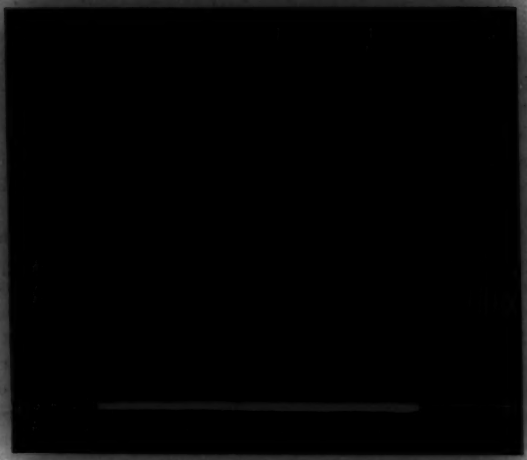

Fig. 12. × ½. Stimulation of left nerve C.D. 140 mm. Fr. 42.
Contraction of left auricle checked by a stop.

But the above effects are only seen at the commencement of an experiment, since there is a progressive change in the response to nerve stimulation on the part of the ventricle dependent on the length of time after dissection or on the strength and duration of previous stimuli. At a later period in an experiment after a long continued stimulus, on its cessation the increase in ventricular tonus or in the height of the ventricular beats may be very great (see Figs. 13, 14) and is often followed by a cessation of beats for a time. On starting again the ventricular beats are henceforth increased in size, so that there seems to be a permanent diminution of the inhibitory apparatus.

Effect of successive periods of stimulation with the interrupted current. After repeated applications of stimuli of moderate strength and duration, even though the periods of stimulation are separated by intervals of rest, the following effects are progressively seen in response to indirect stimulation. The auricle remains contracted throughout the duration of the stimulus, but the ventricular effect varies.

Fig. 13. $\times \frac{1}{3}$. Upper tracing, Ventricle. Lower tracing, Rt. Auricle. ♀ specimen. Rt. nerve stimulated. C.D. 100 mm. Fr. 47. Contraction of right auricle checked by stop.

(*a*) The ventricle may be wholly or partly inhibited throughout the duration of the stimulus but on its cessation shows a staircase effect, while the auricle relaxes (Fig. 13).

Fig. 14. $\times \frac{1}{3}$. Same specimen as in Fig. 13. Same stimulus. Later. See Fig. 15.

Fig. 15. $\times \frac{1}{3}$. Same specimen as in Figs. 13 and 14. Same stimulus. Later.

(*b*) The ventricle may be first inhibited and then gives contractions increasing in height, during the passage of the stimulus (Fig. 14).

(*c*) The ventricle may be unaffected by the stimulus.

(*d*) The size of the ventricular beats may be increased during the stimulus (Fig. 15).

Most of these varieties are evidently a combination of effects already observed in the isolated auricle and ventricle respectively and other combinations are no doubt theoretically possible, while some cases are the converse of others. If, as in variety (*d*), the ventricle had ceased to be inhibited by stimulation of the visceral nerve, it is noteworthy that a change of frequency from 47 per second to 95 per second, the strength of stimulation being maintained the same, sometimes evoked again a short inhibition.

Single induction shocks. Single shocks of sufficient strength evoke a simultaneous inhibition of the ventricle and contraction of the auricle, but both effects are smaller than those obtained with the interrupted current.

B. *Resting Heart.*

In the resting heart stimulation with the interrupted current causes in the ventricle a rise of tonus and beats and simultaneously a contraction of the auricle. On cessation of the stimulus the rhythm of the ventricle is increased and sometimes the height of the contractions, while the auricular contraction which has persisted during the stimulation immediately diminishes.

C. *Additional effects observed.*

Electrical stimulation of the visceral nerve on one side was found to have no effect whatever in either males or females upon the auricle of the opposite side, which confirms the observations of Bottazzi[10]. In addition the right nerve in males was found to have no effect either upon the auricle of the same side or upon the ventricle, but in females the right nerve was equally or sometimes more effective than the left upon the ventricle and the homonymous auricle. Mechanical stimuli such as cutting or pinching the nerve appear to be ineffective, evoking no reaction of any kind in either auricle or ventricle; on the other hand such a chemical stimulus as ammonia applied to the nerve causes a reaction in both auricle and ventricle, but the effect is much less marked than with electrical stimuli.

Conclusions and summary.

From the whole of the foregoing observations the following conclusions may be deduced:—

1. There are no separate inhibitory or motor fibres in the visceral nerves.

2. The influence of the visceral nerves varies according to the nature of their terminal apparatus.

3. The nature of the terminal apparatus is dependent upon the anatomical characters of the structures with which the nerve comes into relation, and it can be modified

(*a*) Temporarily by changes in the strength and frequency of the stimulus.

(*b*) Permanently by a functional decline set up by a time factor or by prolonged stimulation.

With reference to the last conclusion Burian[32] has found that the cephalopod nerve trunk readily exhibits the phenomena of fatigue, so that this effect may play some part, but the probability is strongly indicated from a consideration of the above results and from the observations of Rosenzweig[33] that the change in the ventricular response to nerve stimulation is due (1) to the presence of non-striped muscle in the ventricle either mingled with the cardiac muscle or existing as a separate layer, and (2) to the opposite reactions set up in the two types of muscle by stimulation of the nerve. Since the inhibitory action of the nerve upon the rhythmically contractile cardiac muscle would by its nature be more rapidly impaired than its motor action on the non-striped muscle, a ready explanation is thereby given of the progressive change in the ventricular response, while the "staircase" effects would be due to the grafting of the rhythmical contractions of the cardiac muscle upon the tonic contraction of the non-striped muscle.

Further in the resting ventricle, since the nerve is unable to exercise its inhibitory action, the motor action upon the non-striped muscle is alone obtained with a consequent rise of tonus.

A comparison with mammalian vaso-motor nerves gives a basis for explanation of the changes in the reaction of the auricle and ventricle obtained by changes in the strength and frequency of the stimulus. A diminution of tonus (vaso-dilatation) is obtained in the non-striped muscle of arterioles by stimulation of vaso-motor nerves with interrupted

currents of weak intensity or of low frequency, an effect which is exactly similar to that mentioned above in the case of the auricle, while the simultaneous increase in the size of the ventricular beats would be due to a diminution of tonus in the non-striped muscle in the ventricle, with a consequent dilatation of that viscus, permitting an increased amplitude of the rhythmical beats. In view of Ransom's statement that there is very little continuation of the non-striped muscle of the auricle into the ventricle, the structure of the ventricle and of the auriculo-ventricular junction seems to stand in need of re-investigation, and will be dealt with in a future paper.

The results may be summarized as follows:—

In the Octopodidæ both the right and left visceral nerves are functional in females, but only the left in males. The influence of the visceral nerves is to cause, with single induction shocks, inhibition of the ventricle and contraction of the auricle.

Make shocks are more effective than break in causing inhibition of the ventricle, and the stimulus is effective at any period of the cardiac cycle.

The length of inhibition is within limits dependent on the strength of the stimulus.

During the inhibition set up by a single induction shock there is no diminution in excitability of the ventricular muscle to a direct stimulus.

There is no refractory period in the auricle.

Break shocks are more effective than make in causing contraction of the auricle.

The height of contraction is dependent on the strength of the stimulus.

With the interrupted current the visceral nerves, with stimuli of normal strength and duration, exercise an inhibitory action on the ventricle and a motor action on the auricle.

The motor action causes a true tetanus of the auricle.

Inhibition of the ventricle is dependent to a greater extent on the intensity and to a less extent on the duration of the stimulus than is the case in mammals.

The visceral nerves can also exercise a motor action on the ventricle and an inhibitory action on the auricle.

The change in action can be caused by changes in the strength and frequency of the stimulus and by a functional decline dependent upon continuous stimulation.

The visceral nerves exercise a motor effect upon the resting ventricle.

The visceral nerves exercise no action upon the auricle of the opposite side.

Mechanical stimuli are ineffective, and chemical are less effective than electrical.

(I desire to offer my warmest thanks to Professor Dohrn and all the members of the staff of the Zoological Station in Naples, especially to those connected with the Physiological Department, for their valuable assistance and advice in connexion with this research.)

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ON THE RELATION BETWEEN THE ELECTRIC DISTURBANCE IN MUSCLE AND THE PROPAGATION OF THE EXCITED STATE.

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The researches of Aeby[1] showed that in a muscle fibre excited at one point in its length contraction does not begin at all points simultaneously, but is delayed by an interval of time which increases with the distance separating the point examined from that at which the stimulus is applied. Bernstein[2] observed further that the electric disturbance is delayed in the same way; so that if any two points on the length of a muscle are considered the time interval separating contraction at the one point from contraction at the other is equal to the time interval separating the electric changes which occur at the same points. This coincidence of the time-space relations of contraction and of the electric disturbance is usually described in the inaccurate statement that contraction and the electric disturbance are propagated at the same rate. It does not of course follow from the facts observed that either the contractile disturbance or the electric disturbance is propagated at all. They may, as far as this evidence goes, both be strictly localized changes called up at all points of a fibre's length by an entirely different disturbance which is actually propagated along the fibre. The only inference which can be drawn from the coincidence of time relation is that there is a strong probability that the electric and the contractile disturbances are set in motion either by a single separate conducted disturbance or else one by the other. It is improbable in fact that there should be two disturbances propagated independently and yet propagated at the same rate.

[1] Aeby. *Untersuchungen über die Fortpflanzungs-geschwindigkeit der Reizung in der quergestreiften Muskelfasern.* Braunschweig. 1862.

[2] Bernstein. *Untersuchungen über den Erregungsvorgang in Nerven- und Muskelsysteme*, p. 90. Heidelberg. 1871.

If we admit that there is only one propagated disturbance in muscle we may proceed to enquire whether it is the contractile disturbance, the electric disturbance, or neither. That it is not the contractile disturbance is indicated by two lines of evidence. Experiments made by Woolley[1] and myself[2] have determined the change in the rate of propagation produced by a rise of temperature of 10° C. both in muscle and in nerve. If we take from his experiments and from mine all the determinations made within the range of temperature common to both, namely those in which the lower temperature lay between 8° and 10° C., and if we take a single mean value for each muscle or nerve used, we get the following figures:

Nerve. (Keith Lucas.)

Exp.	Velocity at Tn+10 / Velocity at Tn
1.	1·89
2.	1·66
3.	1·65
4.	1·95
5.	1·89
6.	1·61
Mean	1·77

Muscle. (Woolley.)

Exp.	Velocity at Tn+10 / Velocity at Tn
3.	1·63
4.	1·62
5.	1·54
7.	1·46
8.	1·62
10.	1·92
11.	2·13
12.	2·03
Mean	1·74

The agreement is sufficiently close to suggest that probably the propagated disturbance is the same in muscle as in nerve. And if this is so, the contractile change cannot be the propagated disturbance. Other evidence pointing in the same direction is the fact first observed by Bernstein[3] that the electric disturbance begins before the contractile.

The question then remains whether the electric disturbance is itself propagated or is a local change called up at each point on the fibre's length by some other unknown propagated disturbance. The hypothesis that the electric disturbance is the propagated disturbance has been put forward many times since Bernstein's experiments were published. Bernstein[4] made the assumption tacitly in the use of the word "Reizwelle" for the electric disturbance. Hermann[5] gave the hypothesis more definite shape for the case of nerve at any rate.

[1] Woolley. This *Journal*, XXXVII. p. 122. 1908.
[2] Keith Lucas. This *Journal*, XXXVII. p. 112. 1908.
[3] Bernstein, *loc. cit.* p. 60.
[4] Bernstein, *loc. cit.* p. 58.
[5] Hermann. *Hdb. d. Physiol.* II. p. 193. Leipzig. 1879.

Boruttau[1] defended the position that the electric disturbance is essential to the propagation of the excited state against the opposing views of Gotch[2], Herzen[3] and others. And more recently Lillie[4] has put forward a theory of excitation and conduction, and Macdonald[5] a theory of excitation, conduction and contraction, to both of which theories it is essential that the electric disturbance is itself the propagated disturbance.

The view that the electric disturbance is propagated means of course that it is the electric disturbance in one part of a fibre's length which is the immediate cause of the same disturbance in a neighbouring part. And if this is so, then the rate at which the disturbance is propagated will depend upon the rate at which it develops in each section of the fibre's length. For we must suppose that the production of a given magnitude of disturbance in one section can result only when that magnitude has been reached in a previous section. If for example the disturbance is so influenced by a change of temperature that at each point it develops in half the normal time, then the time of its propagation to a neighbouring point will be halved, and so the whole rate of propagation will be doubled. But if the electric disturbance is a local change called up at each point by a separate propagated disturbance, then there is no reason why the rate of propagation of the propagated disturbance and the rate of development of the electric disturbance should not vary independently. Such independent variation with change of temperature has already been shown by Woolley[6] for the rate of propagation in muscle and the rate of development of the contractile disturbance. For example in his last five experiments quoted above the effect of temperature on the latent period of contraction as well as on the time of conduction was determined, and whereas the mean change in the time of conduction was from 1 to 1·83, the mean change in the latent period of contraction was from 1 to 3·30.

Here then we have a possible method of testing whether the electric disturbance in muscle is the propagated disturbance. When we change the temperature of a muscle, is the time of development of the electric disturbance changed in the same ratio as the time of propagation? If this proves not to be so, then the electric disturbance is not the

[1] Boruttau. *Arch. f. d. ges. Physiol.* LXXXIV. p. 309. 1901.
[2] Gotch. For references see Boruttau, *loc. cit.*
[3] Herzen. For references see Boruttau, *loc. cit.*
[4] Lillie. *Amer. Journ. Physiol.* XXIV. p. 14. 1909.
[5] Macdonald. *Quart. Journ. Experim. Physiol.* II. p. 5. 1909.
[6] Woolley, *loc. cit.*

propagated disturbance; if it is so, then we have one more piece of evidence in favour of the electric disturbance being the propagated disturbance.

Method.

The experiments which I have made to test this point consisted in recording at different temperatures the electric disturbance in response to a single induction shock in the sartorius muscle of Rana temporaria. The temperatures chosen were 8° C. and 18° C., since this is the range over which we have already some reliable data concerning the effect of temperature change on the rate of conduction in muscle and nerve. The record of the electric disturbance was made with a photographic recording capillary electrometer. This instrument was chosen rather than the string galvanometer because it possesses the absolutely essential property that the photographic records can be accurately analysed so as to yield a true account of the changes of potential difference which take place, whereas this accurate analysis is not possible in the case of the string galvanometer. Since the projection electrometer which I used presents certain new features which have proved of considerable advantage in the easy and rapid working of the instrument, and since it has been used also in other experiments shortly to be published, I shall here describe briefly such parts of it as are of special interest.

General arrangement. The illuminating system presents no point of particular interest. It is an arc lamp and a Zeiss optical bench with the usual condensing lenses and cooling tank. On the same table with these stands an electric motor with an arm rotating in the focal plane of the condensing system, used to mark the ordinate lines (see Fig. 3) on the photographic record. The projection microscope (described below) stands on a separate table formed of three cast iron slabs separated by soft rubber blocks and mounted on three large drain pipes which rest on the concrete floor of the room. A wooden partition separates the outer room, in which those parts of the apparatus already mentioned are contained, from a dark room. An aperture in this partition is covered by a hinged plane mirror which normally reflects the image of the capillary tube and meniscus back to a focussing screen placed close to the projection microscope, but can be turned aside to admit the projected image to the dark room when a photographic record is to be made. This arrangement has proved particularly valuable, since it enables the experimenter to keep the image of the mercury meniscus

in exact focus and adjustment until the moment before the record is to be made.

The dark room contains an electrically maintained tuning fork giving 200 D.V. per sec., an adjustable slit and cylindrical lens of 25 mm. focus, and a slide along which the photographic plate is made to travel. The method used for driving the photographic plate at a uniform speed is described below.

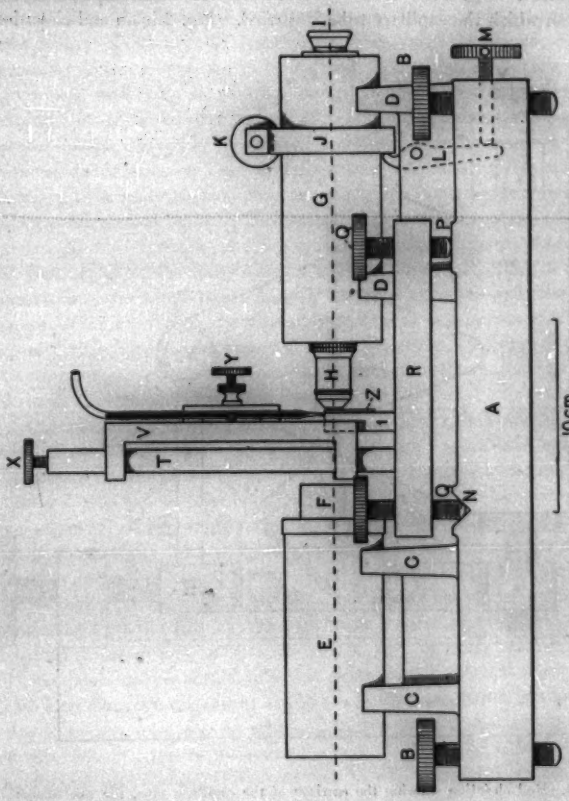

Fig. 1. Side elevation of projection microscope and carriage of capillary tube.

The projection microscope. This was specially designed with two objects in view, first, to afford a very rigid support for the capillary tube and microscope lenses so that the focus and position of the tube might remain unaltered during prolonged experiments, and second, to ensure that the stand on which the capillary tube was carried might be easily removed for the insertion of new tubes or for the changing of the acid, and might be replaced exactly in its original position without elaborate adjustment. Fig. 1 is a side elevation of the microscope with the stand on which the capillary tube is carried. Fig. 2 is an end elevation

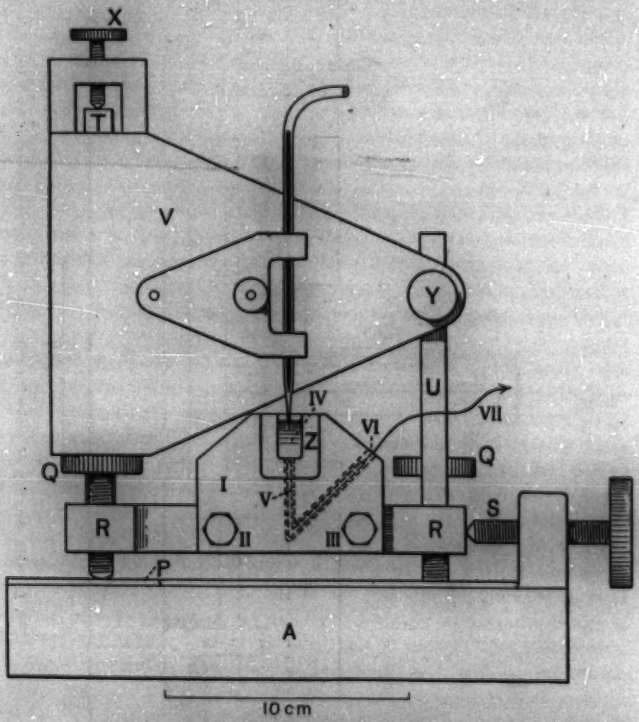

Fig. 2. End elevation showing the carriage of the capillary tube, the microscope tube and its cradles being removed.

showing only the carriage of the capillary tube, the microscope tube having been removed. A heavy cast iron base *A*, carried on three levelling screws *BB*, has projecting from its upper surface the cast iron cradles or V blocks *CC* and *DD* (these cradles are omitted from Fig. 2 in order that the carriage of the capillary tube may be seen). In the cradles *CC* lies a cylindrical brass tube *E*, which carries the condenser *F*. In the cradles *DD* there lies a similar tube *G*, which carries the objective *H* (Zeiss 8 mm. apochromatic) and the eyepiece *I* (Zeiss projection No. 2). On the tube *G* is clamped an adjustable brass ring *J*, which engages with a lever *L* worked by a screw *M*. This screw serves for the fine focussing of the microscope (a spring not shown in the figure keeps *J* in contact with *L*). It will be seen that the tubes *E* and *G* are retained in their cradles by gravity only. Accordingly the whole optical system can easily be removed and replaced in its proper position without disturbance of the capillary tube. The casting *R* carries the capillary tube and the trough of acid in which it dips. The details of these are described below. The casting *R* is carried on three levelling screws *QQ* two of which rest in a groove *N* running transversely across the base *A*, while the third rests on a plane surface *P*. This arrangement allows the whole stand *R*, with the capillary tube and trough, to be adjusted transversely across the optic axis of the microscope by the screw *S* (Fig. 2), or to be removed completely from the base *A* (as for the replacement of capillary tube or acid) without any disturbance of the optical parts of the instrument.

On the casting *R* there are two upright brass rods *T* and *U*, which support the ebonite plate *V* on which the capillary tube is mounted. By means of the screw *X* which rests on the top of the rod *T*, the capillary tube can be raised and lowered in the field of the microscope; the screw *Y* which presses against the front of the rod *U* serves to move the capillary tube along the optic axis of the microscope and to bring it into close contact with the cover glass of the acid bath into which it dips (a spring not shown in the figure keeps the point of *Y* pressed against the rod *U*). The acid bath consists of a block of ebonite I bolted to the casting *R* by bolts II and III. A notch IV is cut out of this block and covered with a thin cover glass *Z* cemented on to each side with pure gutta-percha applied hot. I have never known this cement to give way or to be damaged by the acid. From the lower end of this notch there is drilled a hole V, which meets a second hole opening on the surface of the ebonite at VI. The passage formed by these holes is filled with mercury and the notch IV with dilute

sulphuric acid 1 : 6. The electric connection is made by a platinum wire VII, which dips into the mercury at VI, and so is completely free from contamination by the acid. The arrangements for putting pressure on the mercury in the capillary tube and for making connection with the upper mercury call for no special mention.

The capillary tube. This was drawn by means of the machine which I have recently described[1]. The tube actually used in the present experiment gave an excursion of 12·6 mm. on the photographic plate for a potential difference of ·0086 volt, and reached half the full excursion in ·0087 sec., when the muscle and the non-polarisable

Fig. 3. A typical excursion obtained from the sartorius muscle excited by a single induction shock. Between the beginning of the excursion and the ordinate line preceding it there is a slight displacement of the mercury due to escape of the exciting current. Read from right to left.

electrodes were in circuit with the electrometer. The pressure required to bring the mercury to the working part of the tube was about 22 cm. of mercury. A typical record of an excursion produced by a sartorius muscle is reproduced in Fig. 3 where the tuning fork gives 200 D.V. per sec.

The method of moving the photographic plate. This is shown in Fig. 4. On a table *A*, secured to the wall of the dark room, there are

[1] *Proc. Physiol. Soc.* p. xxxviii. This *Journal*, xxxvii. 1908.

two parallel steel rods *BB* carried in a wooden frame. Along these rods there is free to slide the carriage *C*, on which clips are provided for holding the photographic plate. From *C* to the lever *D* there runs a light steel connecting-rod *E*. The bell-crank lever *D* is pivoted at *G* in the framework *F* (which is attached to the floor and to the table). The short horizontal arm of *D* carries a large lead weight *H* and a connecting-rod *J*. The lower end of *J* is attached to a piston within the oil cylinder *K*. A leak is provided in the piston, so that when the detent *M* is lowered and disengages the pin *N*, the weight *H* forces the piston down into the oil cylinder and so causes the carriage *C* to travel at a uniform speed along the rods *BB*. The rate of leak through the

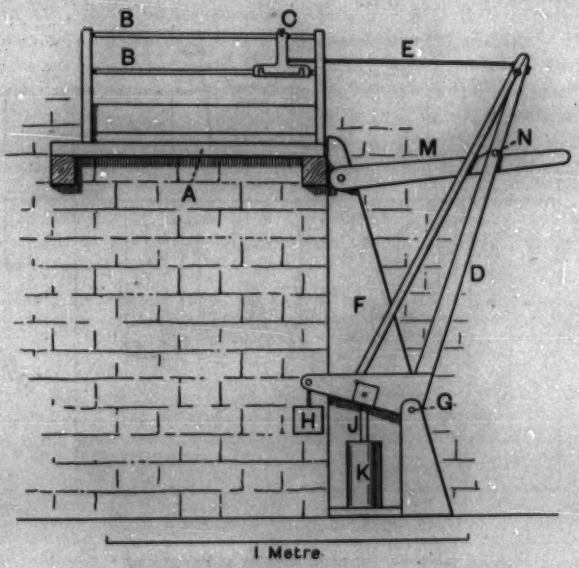

Fig. 4. The mechanism used for moving the photographic plate.

piston (and consequently the speed of the carriage) is controlled by a screw valve, which allows a range of speed for the carriage between half a metre and a fraction of a millimetre per second. The advantages

of this system are its wide range of speed and the rapidity with which it can be reset and charged with a new plate. The one disadvantage is that the viscosity of the oil has a large temperature coefficient. This is of very little importance in practice since the speed of the plate seldom varies by more than 0·5 % during a day, and is always recorded by the tuning fork.

The muscle chamber and electrodes. These are shown in Fig. 5. The muscle is held on a glass rod *A*, and excited by the platinum electrodes connected with *B* and *B*. The leading-off electrodes are glass tubes filled with Ringer's fluid, plugged above by the filter candles

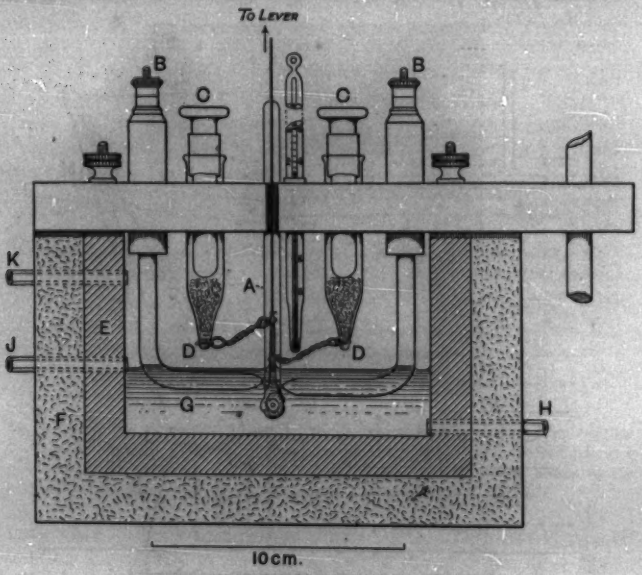

Fig. 5. The moist chamber and electrodes.

CC containing zinc sulphate, and plugged below by the cotton-wool wads *DD* which are connected by moist woollen threads to the muscle. The whole is enclosed in an ebonite trough *E* which has glass sides and is completely covered with thick felt *F*. The lower part of the muscle

is always immersed in Ringer's fluid *G*. When the temperature of the muscle is to be changed fresh Ringer's fluid at the required temperature is run in at *H*, the outlet *J* is closed, and the fluid is allowed to rise to the level of the outlet *K*, so that the muscle is completely immersed. A steady stream is allowed to flow in at *H* and out at *K* until the whole bath has been for some time at the required temperature. The inlet *H* is then closed, and the outlet *J* is opened, when the fluid falls to its normal level, and an observation is immediately taken. I have adopted this method because of the difficulty which I have previously met in trying to bring a muscle to a series of successive different temperatures by means of an air bath. With an air bath each new temperature is taken up so slowly that the progressive changes going on in the tissue assume serious proportions. Also it is extremely difficult to keep the muscle in a satisfactory state of moisture. The present method gives closer concordance than I have previously obtained between observations made at like temperatures before and after a change of temperature. That the tissue is kept in good condition is indicated by the fact that I am usually able to get satisfactory electric responses from a muscle after it has remained set up in the chamber for 48 or 50 hours.

The analysis of records. For this purpose I used a microscopic comparator which I designed some years ago for the measurement of

Fig. 6. Microscopic comparator, ¼th full size.

photographic records of muscular contractions[1]. This instrument is shown in Fig. 6. Sliding on two horizontal steel rods there is a carriage which supports two microscopes. The carriage can be moved along the rods either by hand or by means of a screw placed at the right hand end of one of the rods. The left hand microscope reads on the photographic record, the right hand microscope on a fixed glass scale ruled in tenths of a millimetre. The photographic record rests on a carriage movable along a slide at right angles to that which carries the microscopes. On this carriage there is a circular glass plate which can be rotated in the plane of the microscope field so that the ordinate and abscissa lines of the photographic record may be placed truly parallel to the two main slides of the instrument. For the present purpose the left hand microscope was provided with a goniometer eyepiece (not shown in the figure) for reading the inclination of the electrometer curves. The cross wire of this eyepiece was carefully set on one of the ordinate lines (see Fig. 3) of the record while the goniometer read zero. Then at each half, quarter, or eighth of a double vibration of the tuning fork record the cross wire of the eyepiece was set tangential to the electrometer curve, the angle was read on the goniometer and the corresponding tangent was found from a table. At each setting the linear displacement of the mercury from its zero position was also read from the scale seen in the right hand microscope. The working out of the actual curve of potential difference from these data demands of course a calibration curve of the electrometer with a known P.D. In the present experiment it was found necessary to take a fresh calibration curve for every temperature to which the muscle was brought. For the change of temperature means a change of resistance in the muscle and a consequent increase or decrease in the rapidity with which the mercury will move to its final position after a change of P.D. This precaution was by no means negligible, since with a change of 10° C. in the muscle and electrodes the constant by which the tangents to the electrometer curve must be multiplied to yield a true correction was changed in the ratio of about 5 : 7.

[1] This instrument was used in the investigations recorded in the following papers, where brief mention has been made of it: Keith Lucas, This *Journal*, XXXIII. p. 126. 1905; XXXVII. p. 117. 1908; XXXVIII. p. 118. 1909. V. J. Woolley, *Ibid.* XXXVII. p. 124. 1908. G. R. Mines, *Ibid.* p. 410.

Results.

The first experiments which I made were directed to obtaining a preliminary value for the temperature coefficient of the time of development of the electric disturbance in the sartorius muscle with the object of finding whether there were even an approximate agreement with the temperature coefficient of conduction. For this purpose photographic records were made of the monophasic response of a sartorius muscle excited in the nerve free pelvic end by a single induction shock from a coreless induction coil[1]. The records were taken at the temperatures 18°, 8°, 18° and 8° C. in the order given, and a fresh calibration curve of the electrometer was taken at each temperature. The records were analysed and plotted on squared paper, and measurement was made in each of the plotted curves of the time occupied by the P.D. in rising from 1/10 of its maximum value to its maximum value. It is necessary to take some arbitrary point such as 1/10 of the full value, since it is not practicable to determine the exact point at which the curves begin to rise from zero.

The analysis of Exp. 1 *a* is given below in full as a sample to show the exact method of working. The apparently arbitrary times at which the measurements are given are arrived at in the following way. The summit of the tuning fork vibration immediately preceding the excursion of the mercury caused by the spread of the exciting current (see Fig. 3) is taken as zero. The time of the stimulus is then given as time after that zero. The measurements of the electrometer curve are made at every $\frac{1}{8}$, $\frac{1}{4}$ or $\frac{1}{2}$ of a double vibration of the tuning fork record, and are referred to the same zero.

Exp. 1 *a* at 18° C.

From the calibration curve it is found that

(1) 12·6 mm. excursion = ·0086 volt.

∴ 1 „ „ = ·00068 volt.

(2) At $\frac{1}{2}$ of full excursion inclination of electrometer curve = 48·3°.

Tan 48·3 = 1·12.

$\frac{1}{2}$ of 12·6 = 6·3.

$$\frac{6{\cdot}3}{1{\cdot}12} = 5{\cdot}6.$$

∴ Constant for reducing tangent of angle of inclination to mm. excursion = 5·6.

[1] The muscle was damaged at the tibial end by immersion in hot Ringer's fluid, and led off from the tibial end and from a point about 8 mm. from the pelvic end.

Time from zero	Excursion of meniscus (=h)	Angle of inclination of curve (=θ)	Tan θ	Tan θ×5·6 (=h′)	h+h′	(h+h′)×·0068 =P.D. in volts
·003		stimulus				
·0056	·2	14°	·25	1·4	1·6	·0011
·0062	·5	52	1·28	7·2	7·7	·0052
·0069	1·4	75·5	3·87	21·6	23·0	·0156
·0075	2·5	79	5·14	28·8	31·3	·0213
·0081	4·2	80·3	5·85	32·8	37·0	·0251
·0087	5·6	78	4·70	26·3	31·9	·0217
·0094	6·6	69	2·60	14·6	21·2	·0144
·0100	7·2	57	1·54	8·6	15·8	·0107
·0112	7·7	22	·40	2·2	9·9	·0067
·0125	7·9	0	0	0	7·9	·0054
·0150	7·7	−7	−·12	−0·7	7·0	·0048

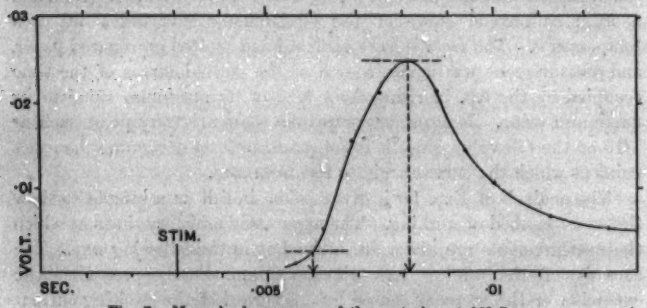

Fig. 7. Monophasic response of the sartorius at 18° C.

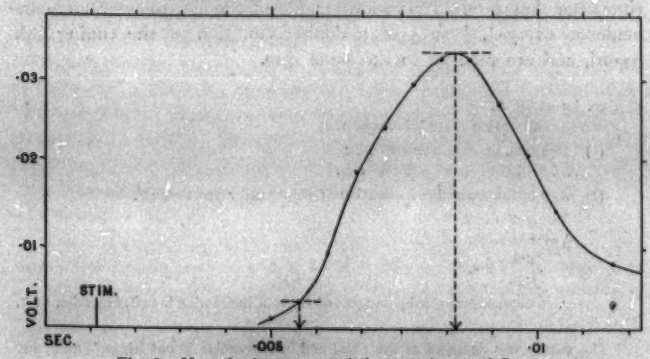

Fig. 8. Monophasic response of the sartorius at 8° C.

For the curves 1 *b*, 1 *c* and 1 *d* I give only the results of analysis in seconds and volts, since the method of working was identical with that used in the case given in full above.

1 *b* at 8° C.		1 *c* at 18° C.		1 *d* at 8° C.	
Time from zero (sec.)	P.D. (volts)	Time from zero (sec.)	P.D. (volts)	Time from zero (sec.)	P.D. (volts)
·0011	stimulus	·0025	stimulus	·00075	stimulus
·0050	·0013	·0050	·0007	·0044	·0007
·0056	·0032	·0056	·0031	·0050	·0017
·0062	·0089	·0062	·0150	·0056	·0046
·0069	·0187	·0069	·0222	·0062	·0134
·0075	·0239	·0075	·0254	·0069	·0202
·0081	·0292	·0081	·0216	·0075	·0255
·0087	·0322	·0087	·0103	·0081	·0333
·0094	·0325	·0094	·0069	·0087	·0364
·0100	·0268	·0100	·0058	·0094	·0360
·0106	·0206			·0100	·0312
·0112	·0140			·0106	·0224
·0125	·0070			·0112	·0138
				·0125	·0082

In Figs. 7 and 8 there are reproduced the first two of the plotted curves from this experiment (Exp. 1, *a* and *b*) showing the changes of potential at 18° and at 8° C. respectively. Broken lines are carried down on to the abscissa from the 1/10 and maximum values of the curve, and show the times between 1/10 and maximum values to have been in 1 *a* ·0021 sec., and in 1 *b* ·0034 sec. The similar curves 1 *c* and 1 *d*, which are not reproduced graphically, gave the times ·0021 sec. and ·0036 sec. respectively. Tabulating these results we have:

Exp. 1.

Observation	Temp.	Time 1/10 to summit	Time at 8° C. / Time at 18° C.
1 *a*	18° C.	·0021 second	
1 *b*	8° C.	·0034 ,,	1·62
1 *c*	18° C.	·0021 ,,	1·67
1 *d*	8° C.	·0036 ,,	

From these figures we get two values for the quotient $\frac{\text{time at 8° C.}}{\text{time at 18° C.}}$, namely *b* divided by the mean of *a* and *c*, which is 1·62, and the mean of *b* and *d* divided by *c*, which is 1·67.

These values obviously fall within the range of variation found by Woolley for the quotient $\frac{\text{time of conduction at Tn}}{\text{time of conduction at Tn} + 10}$. In fact the only experiment which Woolley has at the actual temperatures 8° and

18° C. gives for the quotient the values 1·52, 1·53, 1·61, 1·71, which agree closely enough with the present figures. But Woolley found the values ranging from 1·46 to 2·17 in his experiments made at 10°—20° C., so that if we are to draw any reliable conclusions as to the identity of the quotient for the time of development of the electric change with that for the time of conduction, it is clear that we must obtain the two quotients for comparison from the same muscle under the same conditions. Fortunately this can be done by a slight modification of the experiment already described. If instead of recording the monophasic electric change in the muscle we leave the muscle wholly undamaged so that the change occurs at both electrodes, then we can measure the time of development of the electric change as before and from the same photographic record obtain the time of conduction between the two electrodes by measuring the interval between the occurrence of corresponding points on the electric change at one electrode and at the other.

The experiments made with this object in view were conducted in a manner identical with that already described, except for the one difference that the diphasic change was recorded instead of the monophasic. Care was taken to place the leading-off electrodes as far apart as possible on the muscle, so that there was no doubt that the electric disturbance under the proximal electrode had culminated and begun to fall before the beginning of the disturbance under the distal electrode. The curves reproduced below show clearly that this condition was satisfied.

In the first experiment made in this way five observations were made, namely 2 *a*, *b*, *c*, *d* and *e* at the temperatures 18°, 8°, 18°, 8° and 18° C. respectively. The analysed results of these observations are given in the table below. There was not sufficient escape of the exciting current to mark the time of stimulation with quite the same degree of accuracy as in Exp. 1. However, this is of no importance for the present purpose, as none of the measurements involve the time of stimulation. The time of stimulation is given in each case as nearly as it could be determined.

The analysed results of experiments 2 *a* and 2 *b* are plotted in Figs. 9 and 10. In the figures broken lines are carried down to the abscissa from 1/10 and from the summit of the first phase as before, the interval between these giving the time of development of the electric change. A line is also carried down to the abscissa from the summit of the second phase, and the interval between the summit of the

2 *a* at 18° C.

Time from zero (sec.)		P.D. (volts)
·008		stimulus
·0081	+	·0012
·0087	+	·0046
·0094	+	·0109
·0100	+	·0309
·0106	+	·0421
·0112	+	·0444
·0119	+	·0407
·0125	+	·0292
·0137	+	·0188
·0150	+	·0140
·0162	+	·0088
·0169	+	·0032
·0175	−	·0025
·0181	−	·0101
·0187	−	·0423
·0194	−	·0473
·0200	−	·0453
·0206	−	·0296
·0212	−	·0157
·0225	−	·0056
·0237	−	·0033
·0250	−	·0012

2 *b* at 8° C.

Time from zero (sec.)		P.D. (volts)
·004		stimulus
·0125	+	·0007
·0131	+	·0016
·0137	+	·0043
·0144	+	·0097
·0150	+	·0168
·0156	+	·0278
·0162	+	·0390
·0169	+	·0480
·0175	+	·0533
·0181	+	·0546
·0187	+	·0508
·0194	+	·0445
·0200	+	·0386
·0212	+	·0266
·0225	+	·0164
·0237	+	·0118
·0250	+	·0084
·0262	+	·0037
·0275	−	·0037
·0287	−	·0236
·0300	−	·0444
·0306	−	·0601
·0312	−	·0711
·0319	−	·0693
·0325	−	·0529
·0331	−	·0306
·0337	−	·0231
·0350	−	·0152
·0362	−	·0084
·0375	−	·0059
·0400	−	·0022

2 *c* at 18° C.

Time from zero (sec.)		P.D. (volts)
·008		stimulus
·0081	+	·0014
·0087	+	·0039
·0094	+	·0063
·0100	+	·0146
·0106	+	·0360
·0112	+	·0438
·0119	+	·0479
·0125	+	·0421
·0131	+	·0303
·0137	+	·0202
·0150	+	·0146
·0162	+	·0109
·0169	+	·0075
·0175	+	·0048
·0181	+	·0007
·0187	−	·0109
·0194	−	·0404
·0200	−	·0530
·0206	−	·0546
·0212	−	·0468
·0219	−	·0165
·0225	−	·0063

2 *d* at 8° C.

Time from zero (sec.)		P.D. (volts)
·0047		stimulus
·0119	+	·0010
·0125	+	·0018
·0131	+	·0031
·0137	+	·0040
·0144	+	·0087
·0150	+	·0140
·0156	+	·0237
·0162	+	·0388
·0169	+	·0508
·0175	+	·0566
·0181	+	·0588
·0187	+	·0580
·0194	+	·0510
·0200	+	·0416
·0212	+	·0300
·0225	+	·0176
·0237	+	·0116
·0250	+	·0077
·0262	+	·0061
·0269	+	·0048
·0281	−	·0068
·0287	−	·0189
·0300	−	·0545
·0312	−	·0799
·0319	−	·0818
·0325	−	·0655
·0337	−	·0259
·0350	−	·0139
·0375	−	·0023

2 *e* at 18° C.

Time from zero (sec.)		P.D. (volts.)
·0035		stimulus
·0081	+	·0005
·0087	+	·0018
·0094	+	·0060
·0100	+	·0121
·0106	+	·0259
·0112	+	·0329
·0119	+	·0416
·0125	+	·0405
·0131	+	·0350
·0137	+	·0242
·0150	+	·0160
·0162	+	·0120
·0175	+	·0076
·0181	+	·0029
·0187	−	·0035
·0194	−	·0214
·0200	−	·0450
·0206	−	·0506
·0212	−	·0384
·0219	−	·0249
·0225	−	·0108
·0237	−	·0052
·0250	−	·0025

two phases is taken as a measure of the time of conduction. Exp. 2 *a* shows the time from 1/10 to summit as ·0024 sec. and that from summit to summit as ·0084 sec. The corresponding times from Exp. 2 *b* are

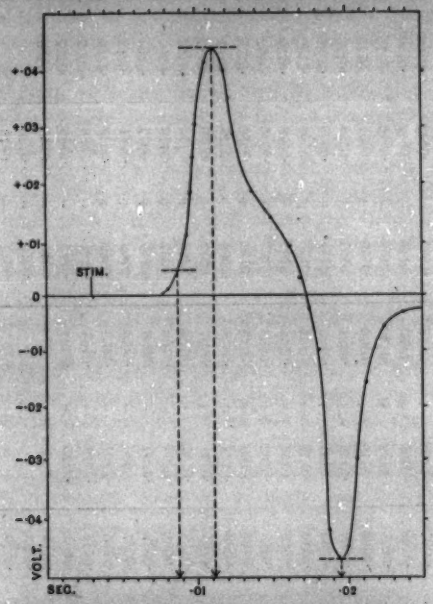

Fig. 9. Diphasic response of the sartorius at 18° C.

·0041 sec. and ·0135 sec. The remaining three curves (2 *c*, 2 *d* and 2 *e*) were plotted in a similar way, and measurements were made, but the curves are not reproduced. The measurements made from them are tabulated below with those already given for the curves 2 *a* and 2 *b*.

Exp. 2.

Observation	Temp.	Time of rise (1/10 to summit)	Time of rise at 8° / Time of rise at 18°	Time of conduction (summit to summit)	Time of conduction at 8° / Time of conduction at 18°
2 *a*	18° C.	·0024 sec.		·0084 sec.	
2 *b*	8° C.	·0041	1·64	·0135	1·59
2 *c*	18° C.	·0026	1·60	·0086	1·57
2 *d*	8° C.	·0042	1·53	·0135	1·59
2 *e*	18° C.	·0029		·0086	

The values for the quotient $\frac{\text{time at } 8^\circ}{\text{time at } 18^\circ}$ are found in the same way as in Exp. 1, each value given being derived from one observation at one temperature and the mean of those at a different temperature immediately preceding and following it. Three values for the quotient are thus found from the five observations. The quotients for the time of rise of the electric change are given in the fourth column, those for the time of conduction in the sixth. There is a close agreement between

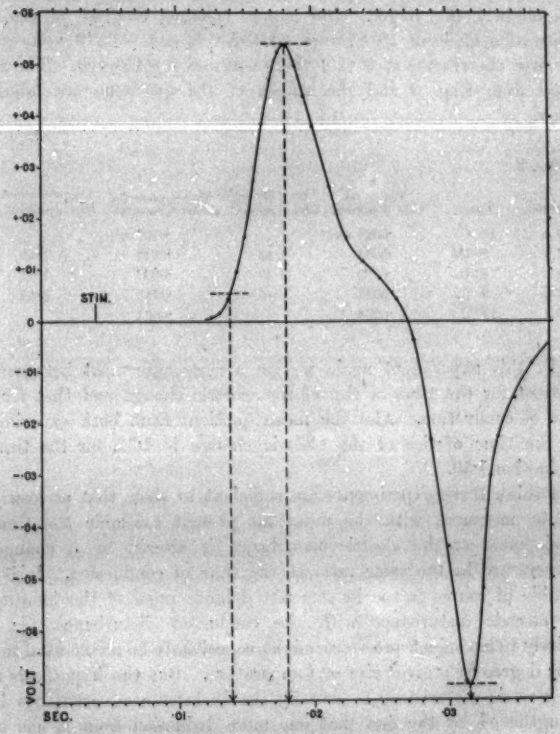

Fig. 10. Diphasic response of the sartorius at 8° C.

the quotients for the two times. They never differ for any set of observations by as much as 4%. The quotients for conduction are steadier than those for the rise of the electric change, but that is probably due to the fact that in the former the time measured is longer and therefore more accurately measured.

A second experiment was made in the same way as Exp. 2. I do not give the full analysis of this experiment (Exp. 3), since it scarcely differed from Exp. 2 except in the detail that whereas in Exp. 2 the electric change under the distal electrode was slightly greater than that under the proximal, in Exp. 3 the change under the proximal electrode was slightly the larger. The actual voltages reached in the first observation made at 18° C. were + ·0434 volt and − ·0379 volt, and in the first observation at 8° C. + ·0569 volt and − ·0479 volt. The times found from Exp. 3 and the values of the quotients are tabulated below.

Exp. 3.

Observation	Temp.	Time of rise (1/10 to summit)	Time of rise at 8° / Time of rise at 18°	Time of conduction (summit to summit)	Time of conduction at 8° / Time of conduction at 18°
3 *a*	18° C.	·0025 sec.		·0101 sec.	
3 *b*	8° C.	·0034	1·45	·0142	1·43
3 *c*	18° C.	·0023	1·48	·0097	1·43
3 *d*	8° C.	·0034	1·45	·0135	1·40
3 *e*	18° C.	·0024		·0095	

In this experiment again we get a close agreement between the quotient for the time of rise of the electric change and that for the time of conduction. Also the mean quotient from both experiments for the time of rise of the electric change is 1·52, for the time of conduction 1·50.

I think these experiments are sufficient to show that as nearly as can be measured with the means at present available the time of development of the electric disturbance is altered by a change of temperature in the same ratio as the time of conduction. It is not possible of course to see in this fact definite proof of the identity of the electric disturbance with the conducted disturbance, for two entirely independent processes might conceivably be accelerated in the same degree by a given rise of temperature. But the hypothesis that the electric disturbance is actually the basis of conduction has been strengthened by the fact that one more deduction from it has been verified experimentally.

Summary.

If the electric disturbance of excited muscle is the basis of propagation of the excited state, then the time of development of the electric disturbance at any point on a muscle should be changed by a given change of temperature in the same ratio as the time of conduction between two points on the muscle's length.

Experiments show that for the temperature change 8° C. to 18° C. in the sartorius muscle of the frog there is close agreement between the changes produced in these two times.

ON THE UNITY OF MOTOR CENTRES.

By Dr M. CAMIS (*of Pisa*).

(*From the Physiological Laboratory, Liverpool.*)

The purpose of the following research was to find if the spinal motor centres are physiological unities. In other words whether the motor neurones which belong to a reflex arc, and which send motor stimuli to a muscle or to a group of muscles, are all involved as a whole in the reflex or may in various cases have a different part from one another, in evoking the motor reflex.

On the first hypothesis the stimuli carried by various afferent paths to a motor station (and causing the same reflex contraction) should always cause the discharge of a motor impulse from the same group of cells; the only differences which we should therefore expect by varying the conditions of stimulation would depend upon the intensity of the stimuli.

On the second hypothesis the nervous impulses carried by various afferent channels to a motor centre should act on different parts of it, impressing on the reflex contraction some peculiar character.

The preparation that I have employed in examining this problem is one which places at disposal three afferent branches and one efferent branch of a reflex arc.

The afferents chosen were *n. saphenus int.* and the two branches (popliteal and peroneal) of the sciatic nerve; the motor nerve was the nerve of *m. semitendinosus*. The muscular contractions were recorded isometrically. To exclude other reflex movements which might confuse the result, the function of all the flexors and extensors of the hip and knee was abolished by severing their nerves or by dividing the muscles from their insertions.

All the experiments were performed on the cat, using the preparation described by Sherrington[1], *i.e.* the decapitated cat in which artificial respiration is maintained. The experiments consisted essentially in comparing the reflex response of the semitendinosus to stimulations of

[1] This *Journal*, xxxviii. p. 375. 1909.

the central stump of each afferent nerve referred to, or to stimulations of two or three of them together.

The arrangement of apparatus is shown in Fig. 1. The rotating drum *A* in its revolutions makes and breaks the primary circuit of the two induction coils; it also breaks with the same rhythm the induction

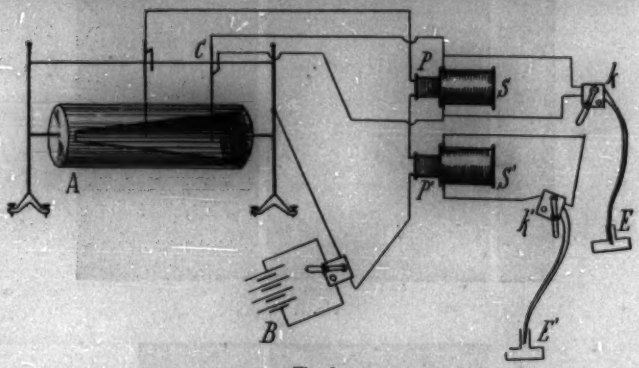

Fig. 1.

current of the secondary coil *S* by means of the key *C*. The secondary coil *S'* can be short-circuited by hand through the key *k'*. With this arrangement the faradisation by the electrode *E'* begins a fraction of a second before that of *E*.

When two simultaneous stimuli are wanted, it is enough to break the connection between *S'* and *C*; both keys *k* and *k'* being open while the primary is not in action the stimuli begin in the same instant and have the same duration as the current in the primary. The electrodes used were of the pattern described by Sherrington[1], and the duration of the stimuli about two seconds.

The maximal stimulus was found for each afferent nerve and the contraction recorded; then maximal stimuli were applied simultaneously to two or three afferent channels. The reflex contraction evoked by simultaneous stimulation of two nerves is stronger than the maximal contraction obtained under the excitatory influence of a single nerve.

Fig. 2 shows the maximal contractions (*A*) obtained by stimulation of the popliteal, and (*B*) by stimulation of the peroneal; $B+A$ is the record of a contraction following simultaneous excitation of both those nerves; it is evidently larger than the others. The last contraction ($A+B+S$) is still larger and is the effect of simultaneous stimulation

[1] *Loc. cit.*

of the peroneal, popliteal and saphenous. This fact has been observed as a constant result when the experiment was performed in the same conditions; I give only another example of it in Fig. 3, where *A* is the

Fig. 2.

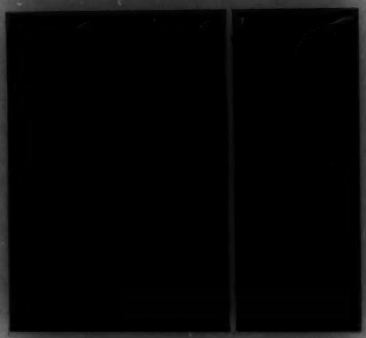

Fig. 3.

maximal popliteal-contraction and *B* the peroneal. *A* + *B* is the effect of both simultaneous stimuli.

If the afferent paths of the reflex arc were all in connection with the same group of motoneurones, this result would be inexplicable; the same may be said if all the motoneurones of the reflex were bound together as a functional unity, so that the excitation of one cell could involve the discharge of the whole centre. Indeed, the stimulus applied to each afferent channel being a maximal one, the addition of another stimulus carried by another channel should be without further effect on the centre.

The experiment gives more complicated results when the excitation of various afferent arcs is not quite simultaneous. I have made experiments in which the interval was about $\frac{1}{10}$ second, so that the second stimulation took place while the muscle was in the ascending phase of the contraction produced by the first stimulus. Sometimes the result is of the same nature as that with simultaneous stimulation, and the descending myogram line (which in my tracings indicates shortening of the muscle) shows a step corresponding to the movement in which the second stimulus is added. But sometimes the double excitation causes a contraction not stronger than that evoked by excitation of a single afferent arc. Fig. 4 shows the myogram $A + B$ of the same height as the myogram A (popliteal contraction) and shorter than B (peroneal).

Fig. 4.

The maximal reflex contraction produced by excitation of the popliteal is as a rule smaller than the maximal evoked by the peroneal nerve. The form too of the myograms is different, and the liminal stimulus is higher for the popliteal branch. I will give further details on this point at another time; now it is sufficient to observe that the different heights of the myograms may give at first sight the impression that in the contraction $A + B$ an inhibitory influence is in action.

Further observation showed that the result just referred to occurs only when the popliteal nerve is the first excited;—a stimulation of the peroneal immediately followed by stimulation of the popliteal gives a contraction higher than each maximal single one. In the experiment furnishing Fig. 5 stimulation of the popliteal followed by stimulation of the peroneal gives a myogram $(A + B)$ of the same height as A (popliteal); but when the peroneal was stimulated first $(B + A)$, the contraction was higher than B[1].

[1] The difference between $B + A$ and B in Fig. 5 is only 2·5 mm.; but a small increase on the top of a curve recorded by an isometric lever means a remarkable increase in the strength of the contraction.

The combined stimulation of the *n. saphenus* and one sciatic branch gives the following results. Precurrent stimulation of the popliteal does not give evidence of summation in the contraction; precurrent stimulation of the saphenous nerve evokes a remarkable *supermaximal* contraction. Fig. 6 shows in $A+S$ the effect of precurrent popliteal stimulation and in $S+A$ the precurrent saphenous excitation.

Fig. 5.

Fig. 6.

Fig. 7.

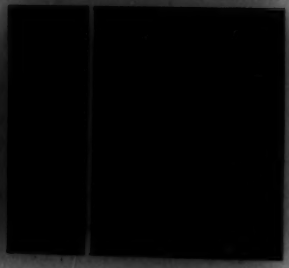

Fig. 8.

The maximal peroneal contraction can be added to the maximal saphenous contraction, not only when the peroneal is excited after the saphenous, as shown by Fig. 7, but even in the opposite case. In the experiment furnishing Fig. 8, *A* and *S* are the contractions evoked by the single stimulus of peroneal and saphenous nerves; $A+S$ is the myogram after combined excitation, the peroneal being the precurrent.

The fact that combined excitation of two afferent arcs may increase the motor discharge caused by the maximal excitation of one arc, indicates that the latter does not put in action all the motoneurones of the centre, and that in the centre there are several portions which act independently of one another. The independence is however not complete. The group of motoneurones which is in direct connection with the afferent channels of the popliteal nerve seems to have a prepotent influence in the function of the centre. When those cells are in action an immediately subsequent stimulus carried by another afferent channel is unable to affect the centre.

In investigating this problem I have employed another procedure consisting in the combination of two subliminal stimuli. The subliminal stimulus, *i.e.* the greatest intensity of current which can be used without evoking a reflex contraction, was carefully found for each branch of the sciatic nerve. Both afferent branches were then simultaneously stimulated and the contraction, if any, recorded. The most frequent result is that the combination of two subliminal stimuli is inefficient.

Sometimes however a trace of contraction is visible, by simultaneous application of two subliminal stimuli.

Such a very small reflex is insufficient to show that in the centre a summation takes place of the stimuli carried by different channels. By applying to the same nerve in the same moment both subliminal stimuli, we have a clear idea of the reflex effect that we should obtain if their summation could take place in the centre. In experiment furnishing Fig. 9 after separate (*S* and *Pop.*) and combined ($S+P$) excitation of

Fig. 9.

the popliteal and saphenous, I applied to the popliteal nerve both electrodes with the same intensity of current already used (subliminal); *C* is the contraction obtained[1].

Summary and Remarks.

Maximal simultaneous stimulation of the central end of any two of the three nerves, popliteal, peroneal and saphenous, gives a reflex contraction in the semi-membranosus muscle which is higher than that given by either of the two singly.

When there is an interval of about $\frac{1}{10}$ sec. between the two stimuli there is also summation except when the popliteal is the first nerve stimulated.

Subliminal stimulation of two of the nerves usually gives no contraction, sometimes however there is a very slight contraction.

Both the results with maximal and with subliminal stimuli afford evidence that the cells of a spinal motor centre can be regarded, from a functional point of view, as divided into several independent groups. Such independence however is not absolute. The trace of contraction, which sometimes can be obtained by combining two inefficient stimuli, is too weak when compared with the effect of an addition of those stimuli to indicate that the centre is a physiological unity; but it means that a partial connection exists between each group of cells.

The fact also of the overpowering influence of the precurrent popliteal excitation—on which I do not venture yet any explanation—indicates that the different parts of the centre are ruled by a common discipline.

The degree of connection is different for the various parts. Figs. 1 and 2 show that the contraction evoked by simultaneous stimulation of both sciatic branches is not much larger than the single maximal contractions. More marked is the increase caused by the addition of saphenous stimulation to the popliteal one (Fig. 6). The peroneal is so independent of the saphenous that the myogram obtained by simultaneous excitation of those nerves is equal to the sum of both myograms, and the supermaximal reflex occurs whichever of the nerves be excited first (Figs. 7 and 8).

My most sincere thanks are due to Prof. Sherrington for the kindness and the hospitality which I found in his laboratory and for the invaluable help which he gave me by suggesting the argument of this research and by criticising the results.

[1] The increase seems too great to be due to difference in the electrical resistance of the popliteal as compared with the saphenous nerve-trunk.

ON THE CONTRACTION OF MUSCLE, CHIEFLY IN RELATION TO THE PRESENCE OF 'RECEPTIVE' SUBSTANCES. PART IV. THE EFFECT OF CURARI AND OF SOME OTHER SUBSTANCES ON THE NICOTINE RESPONSE OF THE SARTORIUS AND GASTROCNEMIUS MUSCLES OF THE FROG.

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(*From the Physiological Laboratory, Cambridge.*)

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In 1905 I[1] showed that in some of the muscles of the fowl there is a certain degree of mutual antagonism in the action of nicotine and of curari, inasmuch as curari, if injected first, prevents nicotine in not too large amount from causing contraction: and if injected in sufficient quantity after nicotine, it decreases or abolishes the contraction. I have also mentioned[2] that in the frog and toad, curari prevents a normally effective dose of nicotine from causing contraction, and that it abolishes the cataleptic condition induced by nicotine, and later[3] that it readily

[1] This *Journal*, XXXIII. p. 387. 1905.

[2] *Proc. Roy. Soc.* B, LXXVIII. p. 187. 1906.

[3] *Arch. Intern. de Physiol.* (*Proc. 7th Physiol. Congress*), V. p. 115. 1907.

prevents the twitchings caused by nicotine in the frog and toad. It was not found to stop the contraction caused by 1 p.c. nicotine.

A preliminary account of further results, some of which are more fully dealt with in this paper, was published a short time ago[1].

The question of the action of curari upon the nicotine contraction has also been dealt with by Boehm[2] in the frog, and by Edmunds and Roth[3] in the fowl. The results of Boehm I refer to on p. 278 and those of Edmunds and Roth on p. 289. I may recall one or two fundamental points with regard to the action of nicotine. Its stimulating effect is due to an action on the muscle, and not to an action on the nerve ending[4]. There is no reason to suppose that its nerve-paralysing effect is not also due to an action on the muscle. Up to ·1 p.c. the stimulating effect is confined to the neural region of each muscle fibre and is of two kinds, one leading to the occurrence of quick contractions more or less conducted throughout the muscle fibres, and the other leading to the occurrence of a slow tonic contraction of a more or less local nature.

I need not recount in full the theory I have put forward to account for these facts. But two points in it must be mentioned. First that two special substances[5] at least (receptive substances) are present in the neural region of the muscle, and that nerve impulses can only cause contraction by acting on a receptive substance. Secondly that the receptive substances form more or less easily dissociable compounds. Thus nicotine in combining with these substances tends on the one hand to prevent them from being influenced by nerve impulses, and on the other tends to cause a breakdown in the contractile substance which leads to contraction. Curari in combining with the receptive substances simply prevents them (and so the contractile substance), from being influenced by nerve stimuli.

I shall use the term receptive substance in describing the phenomena of the action of nicotine and curari, although it belongs as yet to the region of theory, because its use enables the phenomena to be described in the shortest and simplest way. Since nicotine ·25 to 1 p.c. causes a shortening of the muscle both in and outside the neural region, a term

[1] *Proc. Physiol. Soc.* p. lxxi. 1909. (This *Journal*, xxxvm.).

[2] *Arch. f. exp. Path. u. Pharm.* LVIII. p. 265. 1908.

[3] *Amer. Journ. Physiol.* XXIII. p. 28. 1908.

[4] Cp. Part III. This *Journal*, XXXVII. p. 285. 1908.

[5] The question whether (on the lines of Bottazzi's theory) the slowly contracting substance is the sarcoplasm, will not be dealt with in this paper.

is required to distinguish the substance so acted on from the receptive substance. I shall speak of it as the general muscle substance.

I have made observations on a considerable number of the muscles of the frog, but chiefly on the sartorius, the flexor carpi radialis and the rectus abdominis. Each of these three has advantages for certain experiments. In this paper I deal mainly with the sartorius, and to a less extent with the gastrocnemius. I may say that the broad results are the same in the other muscles. The general arrangements for taking graphic records have been described in Part II[1]. The Ringer's fluid I have used contained in nearly all cases rather less salts than in my earlier experiments[2]. Both curari and nicotine solutions were made up in Ringer's fluid, and were prepared from stock 1 p.c. solutions kept in well stoppered bottles in the dark.

There are several sources of error in comparing the results obtained in different experiments. The most important is the intrinsic difference in the corresponding muscles of different frogs. In all the experiments given in this paper the frogs used, unless otherwise mentioned, were male English frogs of weight ranging from 18 to 25 grams, usually freshly caught. But considerable differences occur in corresponding muscles of frogs freshly caught and of the same weight; indeed on cutting out the sartorii it may be obvious to the eye that they are different in size and in tint, some being yellower, some whiter than normal. Consequently in drawing conclusions which involve small differences I rely on a comparison of the results on the right and left muscles of the same frog, except where a large number of experiments have been made. The muscles of the two sides do not give a completely accurate basis for comparison. This is especially the case if one muscle is left longer in the body after death than the other, since the irritability of muscles to nicotine diminishes in the body after death, presumably in consequence of the accumulation of acid.

In some recent experiments I find that the difference in the response of the two muscles is less if the muscle preparations are cut out of the body immediately after death and left in Ringer's fluid for some time before they are taken for experiment. In Fig. 1 I give an example of this. The left sartorius (*l*) was placed in Ringer's fluid 6 mins. after death and left in it for 2 hrs. and 22 mins., and then the effect of ·1 p.c. nicotine tried. The right sartorius (*r*) was placed in Ringer's fluid

[1] This *Journal*, XXXVII. p. 165. 1908. The contraction was magnified 8 times.

[2] It consisted of NaCl ·65 %, KCl ·02 %, $CaCl_2$ ·25 %, $NaHCO_3$ ·15 %. The sartorius remained irritable in this for 1½ to 2½ days.

10 mins. after death, and left in it for 2 hrs. and then the effect of ·1 p.c. nicotine tried. Since the muscles are thin, a short stay in Ringer's fluid would probably be sufficient to get rid of the difference caused by one muscle staying longer in the body than the other. This method however did not occur to me till my experiments were finished. The method I have usually employed is to make two pairs of experiments, using for a given observation the muscle first cut out of the body in one pair, and the muscle last cut out in the other pair (cp. Figs. 6, 28, 30). In the Tables, except Table II, the experiments in which the sartorii were taken from the same frog are bracketed.

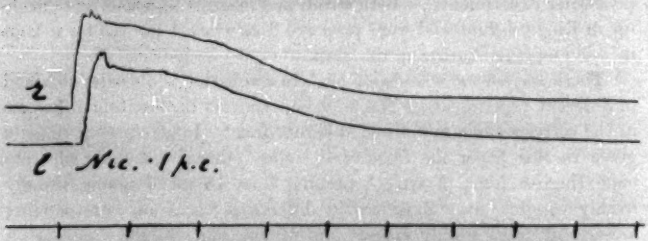

Fig. 1. Sartorius. Description see Text. Load 1½ gram. Time in ½ mins.

A second source of error is the varying temperature at which the experiments are made: other things being equal the response to nicotine increases with the temperature. Most of my experiments were made at a room temperature of 13° to 17° C. I shall mention the temperature when it was 18·5° C. or higher. I have not found any certain difference in the muscles at different seasons of the year except such as can be referred to differences in temperature. A difference in the response to nicotine may occur in the muscles at all seasons.

In determining the degree of tonic contraction caused by nicotine in the sartorius there is sometimes a difficulty in consequence of the twitching. Often it is true, especially in cool weather, the twitching occurs in different fibres at different times, and is not strong enough to appreciably affect the tracing when the muscle is loaded with 1½ to 2 grams. But sometimes, and generally in warm weather, the tracing shows the twitches and the incomplete tetanus simulates tone[1]. This is illustrated in several of the figures, the twitching occurs in an

[1] When a muscle is placed in NaCl ·6, it often remains bent for some minutes after a bout of twitching.

exaggerated form after immersion in NaCl ·6 (cp. Figs. 23, 24). Moreover the sudden fall of the lever after a strong twitch may stretch the muscle and reduce the tone.

Lastly, as I have mentioned in Part I (p. 365), there may be twitching on immersing the muscle in or removing it from a fluid. This however is rare.

In referring in the following pages to the immersion of the muscle in successive solutions of nicotine of increasing strength, it will be understood that the procedure, unless otherwise mentioned, was as follows: the muscle was immersed in each nicotine solution for two minutes, and the interval between running off one solution and pouring in the next was one minute[1].

The Sartorius.

I. The action of nicotine on the normal muscle.

The general behaviour of the sartorius with nicotine has been described in Part I. Since writing that account I have made many further observations, and I give here some additions and corrections.

1. *The forms of curve given by different percentages of nicotine.* The minimal percentage of nicotine which causes a visible contraction by the graphic method (load 1½ grams) is, as I have said earlier (*op. cit.*), about ·001 p.c. but ·001 p.c. does not always cause contraction. The minimal percentage, although in part depending upon the condition of the muscle, in part depends upon the temperature. Thus in warm

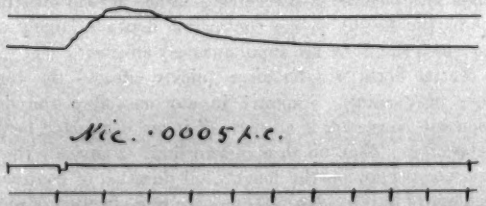

Fig. 2. The sartorius was left in Ringer's fluid at 22°—23° C. for 55 mins. For 50 mins. there was gradual extension, the straight line shows the position of the lever at the beginning. Then the muscle was immersed in nicotine ·0005 p.c. at 22° C. Time in ½ mins. Load 1½ grams.

[1] Usually the muscle was stimulated with a make and break induction shock before applying the first nicotine solution.

summer weather with the fluids at a temperature of 22°—23° C., a contraction may be given with less than ·0005 p.c. (cp. Fig. 2).

The sartorius[1] is not a satisfactory muscle on which to determine the relation between the height of the tonic contraction and the percentage of nicotine since the fibrillar twitches caused simultaneously modify the curve (cp. above p. 238). Nevertheless the main facts are, I think, clear.

With percentages of nicotine which affect the neural region only, i.e. ·001 to ·1 p.c., the height of the tonic contraction increases with the percentage. This is shown in Fig. 3.

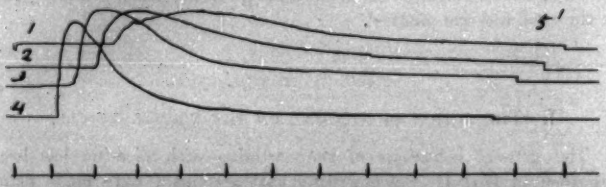

Fig. 3. (1) Right sartorius in ·001 p.c. nicotine. (2) Left sartorius of same frog in ·01 p.c. nicotine. (3) Right sartorius of another frog in ·01 p.c. nicotine. (4) Left sartorius of same frog in ·1 p.c. nicotine. Each muscle was left in Ringer's fluid for 5 mins. before immersion in nicotine. After 5 mins. the drum was stopped for 5 mins. Load 1½ gram. Time in ½ min. (The difference in the height of contraction of 2 and 3 was probably not due to a difference in the muscles, but to 2 having been left rather longer in the body after death.)

The time which elapses before a fall in the curve begins, is in general less with increase of percentage. Since in each fibre relaxation soon sets in, the height of the contraction depends largely upon the degree to which the fibres are simultaneously affected.

The central fibres will be more quickly affected the higher the percentage of nicotine. Similarly in any one fibre the degree of contraction will be greater if all parts of it are contracted before there is any relaxation. Thus up to a certain limit at any rate the relation mentioned above between the height and duration of contraction and the percentage of nicotine depends upon the rate of action of nicotine on the total receptive substance.

With percentages above ·1, the primary slow contraction has two components, viz. the contraction in the neural region due to stimulation

[1] The rectus abdominis is perhaps the best muscle for the purpose. I shall give an account of this later.

of the receptive substance, and a contraction more or less throughout the muscle, including the neural region. In Part I, I stated that the primary rise was comparatively slight in passing from ·1 to 1 p.c. In later experiments in which the muscles of the two sides of the same frog have been compared, I find that the rise with 1 p.c. is always much greater than with ·5 p.c.[1] (Fig. 4).

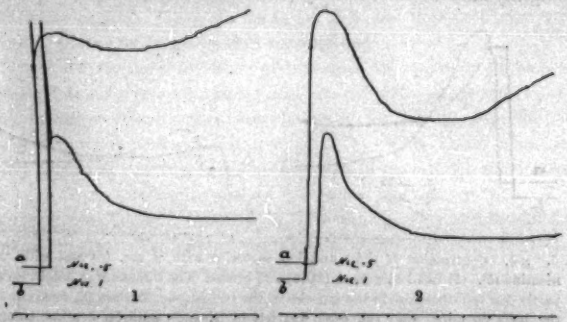

Fig. 4. ×½. (1) Contraction of right and left sartorius (*a*) with ·5 p.c nicotine, (*b*) with 1 p.c. nicotine. Load 1½ grams. (2) A similar experiment with a load of 2½ grams. Time in ½ mins.

In all these cases the height of the contraction is above the average.

But the difference between the height of the primary rise caused by ·1 and by ·5 p.c. nicotine is comparatively slight and if the muscle treated with ·5 p.c. nicotine is taken out of the body about half an hour after that treated with ·1 p.c. nicotine, the decrease in irritability which occurs in the former is often sufficient to make the height of the contractions approximately equal (cp. Fig. 5).

Since ·5 p.c. nicotine acts on the general muscle substance as well as on the receptive substance, the difference between its effect and that of ·1 p.c. nicotine must be due in part if not altogether to this additional action[2]. We have seen above that the increasing effect of nicotine up to ·1 p.c. is largely due to summation of contractions. We may infer then that when the percentage of nicotine is sufficient to cause complete

[1] The error in the earlier experiments was probably partly due to the stronger solutions of nicotine being used several times.

[2] It is possible that the direct action of strong nicotine in the general muscle substance in the neural region reduces the effect due to stimulation of the receptive substance.

summation, differences in rate of action on the receptive substance have little if any effect.

The much greater effect of 1 p.c. than of ·5 p.c. nicotine, I take to be due to its much greater action on the general muscle substance.

The form of the curve depends of course upon the load. Even when the difference is only between 1 and 2½ gr., all parts of the curve are slightly affected but the early part of relaxation more than the rest.

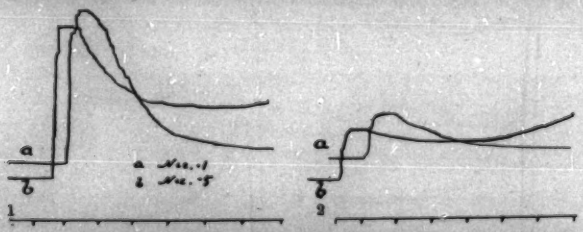

Fig. 5. ×⅓. Contraction of right and left sartorius with ·1 p.c. (*a*) and with ·5 p.c. nicotine (*b*). (1) Load 1½ grams, (2) load 2½ grams. The difference in height no doubt partly due to differences in the muscles of the two frogs. Muscles (*b*) were cut out of the body 30 mins. after (*a*), each was left in Ringer's fluid for 5 mins. before immersion in nicotine.

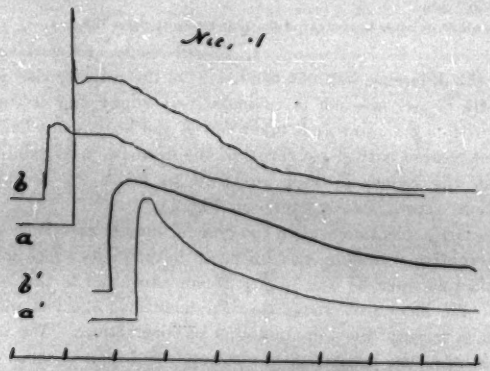

Fig. 6. Two pairs of sartorius muscles immersed in ·1 p.c. nicotine.

a	Load 1	gram.	8′	after pithing	frog.	
b	,, 2½	,,	28′	,,	,,	,,
a′	,, 2½	,,	12′	,,	,,	,,
b′	,, 1	,,	35′	,,	,,	,,

In making the comparison, account must be taken of the decrease of irritability in the muscle left longest in the body. This can be done by using the light load for the muscle first cut out in one experiment, and for that cut out second in another. An example is given in Fig. 6 of the effect of ·1 p.c. nicotine in these circumstances, though it must be mentioned that the difference between *a* and *b* is greater than in the other experiments I have made.

With a load of only $\frac{1}{4}$ gram, there may be, though there is not always, a prolonged considerable contracture.

When the stronger solutions of nicotine are employed (·5 to 1 p.c.) the fall in the curve soon passes into the rise of nicotine rigor. With ·5 p.c. nicotine (the temperature being under 17° C.) the rigor rise usually begins in 3 to 4 minutes (Fig. 7), with 1 p.c. nicotine it usually begins in about a minute (Fig. 4) and goes much more rapidly than with ·5 p.c. It begins sooner the higher the temperature. There is however considerable variation in different muscles independently of the temperature. The rate of rigor rise is affected by slight variations in the load, thus it is slower with a load of 4 grams than with one of 2 grams. The curve of rigor rise is at first concave to the abscissa but soon becomes convex.

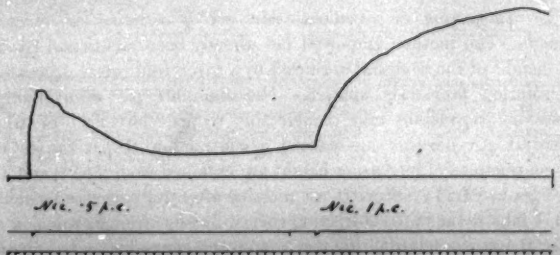

Fig. 7. × $\frac{1}{3}$. Convex curve of rigor rise with 1 p.c. nicotine after the sartorius has been in ·5 p.c. for 5 mins. Load 3$\frac{1}{2}$ grams. Time in 10 secs.

The rigor rise is largely if not wholly dependent upon continuous immersion in nicotine; if the nicotine solution is run off, there is a slight fall in the curve although nicotine still adheres to the muscle; how long this lasts I have not determined. A fall in the rigor curve is always obtained by washing out the nicotine with Ringer's fluid (Fig. 8), though the fall is a slow one.

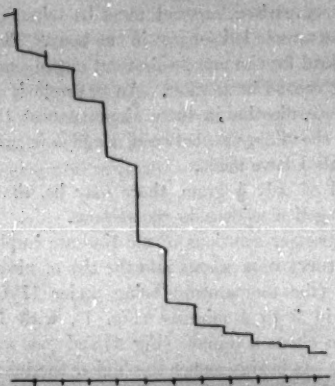

Fig. 8. × ½. The sartorius had been 4½ minutes in 1 p.c. nicotine. The line of the signal marker shows the position of the lever at the beginning. The drum was then stopped, Ringer's fluid substituted for nicotine, and at the end of 5 mins. a tracing taken for 30 secs. The drum was again stopped, fresh Ringer's fluid added, and at the end of 5 mins., a tracing taken for 30 secs., and so on for 55 mins. Each down-stroke of the marker indicates an interval of 5 mins.

2. *Immersion in successive solutions of nicotine of increasing strength.* The method employed has already been mentioned (p. 239). The height of the contraction caused by a given solution is decreased by the solution previously applied. Nicotine ·001 p.c. even though it causes no contraction may considerably reduce the effect of ·01 p.c. After ·01 p.c. nicotine has caused a contraction, ·1 p.c. causes none, but contractions of increasing height are obtained from ·25, ·5, and 1 p.c. (cp. Figs. in Part I). Since ·1 p.c. nicotine after ·1 p.c. causes no contraction, I take it that the contraction caused by any stronger solution after ·01 or ·1 p.c. is solely due to an action on the general muscle substance. Nicotine ·25 and ·5 p.c. after the more dilute solutions cause a moderately quick primary rise which is usually maintained for 2 to 3 minutes with little or no change (cp. Figs. in Part I), and then passes into the rigor rise.

Nicotine 1 p.c. causes a primary rise which is higher than that caused by any of the diluter solutions; the rise is sometimes followed by a slight fall, sometimes it continues for a short time at a much slower rate than previously and at a slower rate than subsequently. If the time of immersion of the muscle in ·5 p.c. nicotine is decreased, the

primary rise with 1 p.c. nicotine is usually followed by a fall; if it is increased to 5 or 6 minutes, there may be no primary rise with 1 p.c. but only a smooth curve more or less convex to the abscissa (cp. Fig. 7).

3. *The time factor in the action of dilute nicotine.* I have made only a few experiments on the sartorius with regard to this since the flexor carpi radialis and the rectus abdominis, in which the receptive substance is more developed, are better adapted for the purpose.

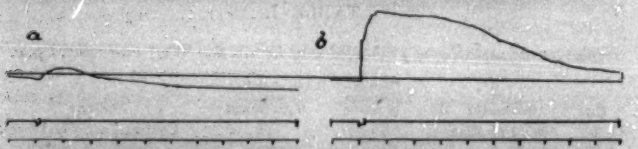

Fig. 9. ×½. Right sartorius muscle in 25 c.c. nicotine ·0001 p.c. for 24 hrs. then in nicotine ·1 p.c. (*a*). Left sartorius in 25 c.c. Ringer's fluid for 24 hours, then in nicotine ·1 p.c. (*b*). Load 1½ grams. Time in ½ mins.

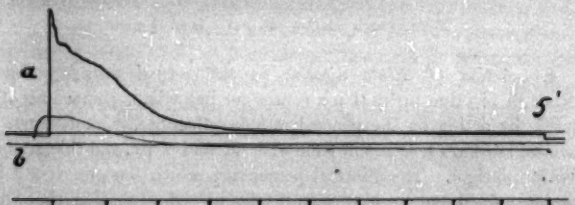

Fig. 10. Right and left sartorius in nicotine ·0001 p.c. (*a*) 1 hour, (*b*) 5 hours, then each in nicotine ·1 p.c. Towards end, drum stopped for 5 mins. Load 1¾ grams. Time in ½ mins.

We may take the absence of contraction with ·1 p.c. nicotine as showing a definite stage of action in the receptive substance, and for convenience we may speak of this stage as one of paralysis. We have seen that ·01 p.c. nicotine paralyses (in the sense given above) the receptive substance in 2 to 3 minutes, and that ·001 p.c. in the same time considerably reduces the effect of ·01 p.c. nicotine. If a sartorius is placed for 15 minutes in ·001 p.c. nicotine, and then dropped in ·1 p.c., there is sometimes no effect, sometimes slight twitching without obvious bending of the muscle, i.e. the receptive substance is paralysed or nearly paralysed by ·001 p.c. in about a quarter of an hour.

From the results given below in Table I it appears that it takes more than 24 hours for ·0001 p.c. nicotine to paralyse the receptive substance

(Fig. 9, see also Table III B, Exp. 2) and that very little effect is caused in the same time by ·00001 p.c. nicotine. The solutions however may have been a trifle less than the nominal strength as they were made up from a ·1 p.c. solution which had been prepared some days, and not from the stock 1 p.c. solution. In another experiment the action of ·0001 p.c. nicotine was more marked (Fig. 10).

TABLE I.

Effect on the sartorius of prolonged stay in nicotine ·0001 *and* ·00001 *p. c.*

Exp.	Muscle immersed in	Time of immersion of muscle	Height of contraction in mm. with ·1 p.c. nicotine	Remarks
[1] 1	·00001 nicotine	5 hrs.	21	Contraction normal form.
2	·00001 ,,	24 ,,	23	,, ,, ,,
3	·0001 ,,	24 ,,	3	In 50 secs. falls to base line (Fig. 9 *a*).
4	Ringer's fluid	24 ,,	18	Normal form, but rather slower (Fig. 9 *b*).

25 c.c. of each solution was used. Load 1½ grams.

4. *Action of dilute nicotine on the general muscle substance.* Although nicotine up to ·1 p.c. causes no immediate shortening of the muscle by acting on the general muscle substance, a shortening, as I have said in Part I, occurs in time with ·01 and ·1 p.c. and this gradually passes into rigor. The minimal percentage which hastens rigor I have not determined, but we have seen in the preceding section that ·00001 p.c. may have very little effect.

Most of the observations I have made have been on the action of ·1 p.c. nicotine, and the results have varied widely. The variation is partly due to a difference in the muscles; but to a much greater extent to differences of temperature. Thus in winter and spring the sartorius was but little altered from the normal shape and gave a good contraction with induction shocks after a stay of a day in ·1 p.c. nicotine, whilst in some experiments in summer with a room temperature of 22° C., there was rigor in about 6 hours. At this temperature the beginning of the shortening, as shown both by naked eye observation and by the graphic method, began in a quarter to half an hour. Some experiments bearing on this point are given on p. 268. I am inclined to think that at high temperatures, ·1 p.c. nicotine has sometimes a slight immediate effect

[1] As noted in the Introduction, the bracketed experiments in this and in other Tables were made from two sartorii of the same frog.

on the general muscle substance as it also has after a prolonged action of curari (cp. pp. 264, 267).

5. *Naked eye and microscopic observations.* If a fresh sartorius muscle is placed in nicotine, the obviousness of the fibrillar twitches and the time they continue is within limits proportional to the dilution of the nicotine. With ·001 p.c. (in warm weather) the fibrillar twitches go on for 2 to 4 minutes, and there is very little bending or shortening of the muscle to be seen. With ·1 p.c. the muscle becomes bent sharply on itself once or twice, not infrequently first to one surface and then to the other, and rapid local fibrillar twitches are seen for about 30 seconds. The muscle remains bent on itself for a minute or two and then gradually relaxes and becomes again flat but the knee end is a little bent to the lateral side. The time taken for this varies considerably, especially as regards the last stage of relaxation, but usually it is about 5 minutes. This protracted bend of the muscle is always towards the inner surface[1]. It has been shown by Grützner that there is a thin layer of smaller fibres on the outer surface of the muscle. The tonic contraction, then produced by nicotine, is considerably greater in the larger fibres of the inner surface than in the smaller ones of the outer surface. It is rather greater in the fibres of the lateral border of the muscle than in those of the medial border; not infrequently this is shown in the bent condition of the muscle by its being slightly twisted.

The changes in form which the muscle undergoes in passing into rigor in dilute nicotine varies in different cases, but there are two chief varieties (1) the bend on the lateral border at the knee end increases, and this end bends towards the inner surface (the bending may gradually spread $\frac{1}{3}$ to $\frac{1}{2}$ way down the muscle, or it may begin a little past the entrance of the nerve); after this has gone on some time, the pelvic end bends to the outer surface, so that on a side view the muscle is *S* shaped; as these changes are going on there are small bulgings and depressions formed on the whole surface, then the muscle shrinks and takes up the definite rigor shape which is one with a fairly symmetrical bend to the inner surface varying in degree with the degree of rigor[2].

[1] I speak of the surface next the skin as outer surface, the surface next the muscles as the inner surface; the border at which the nerve enters as the medial border, the opposite as the lateral border.

[2] Zenneck (*Pflüger's Arch.* LXXVI. p. 54. 1899) describes the sartorius as nearly always bending towards the inner surface, if it bends at all, when placed in strong solutions of various salts. This no doubt was a rigor shortening.

(2) The muscle in the region of the chief nerve branches, and in the knee end, except the extremity, acquires a series of short folds, usually also being bent to the lateral border, similar short folds come later in the pelvic end and the muscle is more or less twisted; the muscle then passes into the rigor form as given above[1]. If the muscle is placed in 1 p.c. nicotine, the rigor form is reached without the intermediate stages, and the shrinking and final bend of the muscle is greater. There are other minor varieties unnecessary to describe, the essential point is that in the slow rigor caused by ·01 to ·1 p.c. nicotine, the different fibres and to a considerable extent different parts of the fibres are acted on at different rates, and thus cause the muscle to undergo a series of changes of form. The muscle, then, does not consist of two classes of fibres only but of fibres with many shades of difference. This is also indicated by the different vitality of the fibres. If the progress of rigor is observed from time to time under a low power of the microscope, it is found that the rupture of some of the fibres begins comparatively early, at a time when induction shocks still give a twitch of the whole muscle. As rigor proceeds more and more fibres rupture. The ruptured fibres are more numerous on the inner than on the outer surface, but the change is not confined to either surface.

The different appearances shown by the fibres under the microscope are as follows: (*a*) They have close set deepish depressions and bulgings similar to those seen at the angles of much bent fibres, this I have spoken of as crinkling (Part I, p. 358). (*b*) They have regular smooth bends at short intervals giving them a wavy look, but adjoining fibres are in different states. (*c*) The fibres are folded at right angles to the plane of the muscle, all the fibres in one plane being similarly folded; the distance from crest to crest of the folds varies; this is caused when the muscles in the plane below are as a whole contracted; it sometimes occurs on both surfaces owing to greater contraction of the middle layer. (*d*) Some of the fibres have short folds in the plane of the muscle.

Forms (*a*) and (*b*) are best seen on the outer surface; in the early stages of nicotine action they are most marked in the regions known to contain nerve endings, suggesting that the contraction is greater in the neural region and throws the surrounding non-neural regions into folds. But though they are most marked in these regions, they occur early in all parts of the muscle except the extreme ends which are affected much later, so that the difference in the time of beginning change in neural and non-neural regions if it occurs at all can at most be slight. Form (*d*) chiefly occurs on the inner surface, it is caused by some of the fibres being contracted and others not, or by some being contracted more than others. This crinkling and folding of the fibres can be produced in an exaggerated form by stimulating the muscle with not too strong tetanising currents at a stage when the irritability is much diminished; some only of the fibres contract, the intervening ones are thrown into folds; if the current is increased in strength, the intervening fibres also may contract.

[1] This second series of changes occurs in Ringer's fluid, but the rigor shape is less marked.

In dilute nicotine the fibres which rupture show, as a rule, the approaching change by becoming cloudy (those of the inner surface often showing a clear outer margin); after rupture, the ends shrink. In 1 p. c. nicotine the fibres commonly break at the depressions of the crinklings, forming irregular splits which look like very refractive transverse bands. The breaks in the fibres may begin in any part except the extreme ends.

Grützner describes the fibres of the outer surface as containing more protoplasm and as being less transparent than the rest. In the fresh condition I see little difference, but the fibres of the inner surface are as a whole the first to become cloudy. If Grützner's conclusion is correct, it is the less sarcoplasmic fibres which in this case give the strongest tonic contraction with nicotine. In most of the cases I have investigated the reverse is the case.

For some time after the muscle has assumed the rigor form contraction more or less local can be obtained in parts of the muscle by tetanising currents.

In order to observe the last remains of irritability, the currents must be very strong; currents which are too strong to be borne on the lip may produce no effect, though still stronger currents (1 Dan. sec. coil at 0) may give marked contraction. The relaxation is slow and fatigue is rapid, so that the effect soon decreases or ceases. At a certain stage, the stimulus produces no twitch, but if prolonged the whole of the muscle shortens towards the local wheal formed at the electrodes. If the muscle is stretched, a number of fibres are broken, but contraction is much more easily observed, and it occurs in parts which, in the shrunken muscle, appeared inexcitable.

I have not found any constant difference in the time at which the ends and the rest of the fibres lose their irritability, but in the experiments in which I have paid atttention to the position of the fibres retaining their irritability longest, it was found that on the whole the irritability of the outer surface lasted longer than that of the inner, and that the fibres on the lateral border were the last to die.

II. Action of curari and of its salts on the normal muscle.

The curari I have used is the crude substance obtained in gourds. A 1 p.c. solution was made up in Ringer's fluid from time to time and from this the weaker solutions prepared as they were required. Paralysis of the sciatic was produced in about 1½ hours in a frog weighing 20 grams (with the brain destroyed), by injecting 1 c.c. of a ·05 p.c. solution into the dorsal sac, *i.e.* by ½ mgrm.

I have very little new to say in this section on the effect of curari

but it is important to state certain effects as a preliminary to the next section.

Since curari contains salts, and potassium salts prevent dilute nicotine from causing contraction (cp. p. 275), it is necessary to know what effect the salts of curari have on the sartorius.

A portion of the 1 p.c. curari solution was evaporated to dryness, and the residue heated over a Bunsen burner to destroy the organic substances. This was done in two cases, in one the residue was ground with water, and in the other with a little ·1 p.c. HCl which was subsequently neutralized with Na_2CO_3. Water was added to each corresponding to the quantity evaporated, so that the solution contained the salts present in a 1 p.c. solution of curari. No difference was found in the effect of the two solutions.

The sartorius was placed in one of these solutions, undiluted or diluted with Ringer's fluid, for 15 minutes (unless otherwise mentioned), then immersed in nicotine and the contraction, if any, recorded.

The salt solution diluted with three or more volumes of Ringer's fluid had no effect upon the nicotine response: nicotine ·001 p.c. caused a slight contraction in four out of six cases, *i.e.* at least as often and as great as in the normal muscle, and ·01 p.c. nicotine caused the usual contraction. In one experiment the muscle was left in the salt solution diluted with three volumes of Ringer's fluid for 30 minutes; nicotine ·001 p.c. caused a slight contraction.

The solution diluted with an equal volume of Ringer's fluid had no certain effect upon the contraction caused by ·01 p.c. nicotine (·001 p.c. was not tried). In an experiment the muscle was left in the salt solution for 30 minutes instead of 15; in this case the response to ·01 p.c. was diminished.

The undiluted solution was applied to the muscle for 30 mins., this diminished the response to ·01 p.c. solution, but had no certain effect on the response to ·1 p.c. nicotine. In the two experiments in which ·001 p.c. nicotine was tried, there was no response. The excitability of the muscle to single induction shocks appeared to be more reduced than the excitability to nicotine.

These results show that the salts in a 1 p.c. solution of curari in half-an-hour have little effect on the response of the sartorius muscle to ·1 p.c. nicotine, though they reduce the excitability to more dilute solutions; and that the salts in a ·25 p.c. solution of curari have in the same time no influence on the nicotine response appreciable by the method used.

When a sartorius is placed in curari ·1 to 1 p.c., made up in Ringer's fluid, there is a decrease in its irritability to induction shocks, which comes on sooner and develops more quickly the stronger the solution of curari[1]. Local irritability is not completely lost for 1 to 2 days. The effect, however, may be due to the salts in it, and as the solution is hypertonic, some change would be expected in prolonged action.

The experiments were made in two ways. (*a*) A graphic record was taken, the vessel containing the muscle was filled with a given percentage of curari, left for a given time, then poured off and the muscle stimulated directly with induction shocks, the electrodes being at opposite ends of the muscle; the curari solution poured back, left for another period, stimulated, and so on for several hours. By this method there is a rapid decrease in the height of the contractions in a few minutes even with dilute curari, mainly due to paralysis of the receptive substance. After this there is a slow fall in the height of the contractions and stronger and stronger stimuli are necessary to produce more than a small contraction. The rate of reduction of irritability increases with increase in the percentage of curari from ·1 to ·5 p.c.

(*b*) (March.) Muscles were placed in curari solutions ·1, ·25, ·5 p.c. and 1 p.c., taken out at intervals, placed on glass, and stimulated at different points with induction shocks, the electrodes being about 2 millimetres apart, and the contraction observed with a dissecting microscope. In all the irritability lasted more than a day, but the reduction of irritability was greater the greater the percentage of curari, it was rapid in ·5 to 1 p.c. curari and was accompanied by some shrinking. The decrease of irritability is accompanied by a decrease in conduction, so that the contraction becomes more local. At a later stage there is local contraction only, whatever the strength of the induction shock, and the contraction is polar; the make contraction is equal to or stronger than the break contraction, probably as M. and Mme Lapicque point out because the make current is of longer duration. Fatigue and exhaustion occur rapidly. At this stage there are many ruptured fibres.

III. Effect of curari on the contractions caused by nicotine.

1. *Action of nicotine up to ·1 p.c. on the curarised muscle.*

Since the immediate contraction caused by nicotine up to ·1 p.c. is confined to the neural region, the first point to determine is the percentage of curari which prevents the diluter solutions of nicotine from having an effect. The muscle was immersed in the curari solution, usually before, sometimes after, fixing in the apparatus; it was then washed for 5 to 10 secs. in Ringer's fluid, and finally nicotine poured into the vessel containing it. Successive nicotine solutions were usually applied (cp. p. 239), but the effect of curari is of course best determined by the response of the muscle to the first solution (cp. p. 244).

The results with curari ·0001 to ·001 p.c. are given in Table II.

[1] According to M. and Mme Lapicque (*C. R. Soc. Biol.* p. 991. 1906) the irritability decreases gradually without any sudden break.

TABLE II.

Effect on the action of nicotine ·01 to ·1 p.c. caused by placing the sartorius for 15 to 30 minutes in a solution of curari.

The load in all cases was 1½ to 2 grams. The Exps. were made in all seasons except spring.

Exp.	Temp. of room	Percentage of curari	Number of minutes in curari	Effect of nicotine ·01 p.c.	Effect of nicotine ·1 p.c.
1	—	·0001	15	—	Slight gradual rise of 5 mm. (Fig. 12.)
2	—	·0001	15	Rise 3 mm. and slight twitches	Rise ·5 mm.
3	—	·0001	30	Slow rise ·5 mm.	Slow rise ·5 mm.
4	—	·0001	30	—	Rise 3·5 mm. with one or two slight twitches.
5	—	·0001	30	—	Slow rise 1 mm.
6	—	·0005	15	—	0
7	—	·0005	15	—	Slow rise 1 mm.
8	—	·0005	15	0	Slow rise 1 mm.
9	—	·0005	30	—	Twitch 4 mm. at beginning, no rise or fall after 20 secs.
10	—	·0005	30	—	Slight twitches with rise 3 mm.*
11	—	·0005	30	—	Slow rise ·5 mm.*
12	—	·00075	15	0	No effect for 1 min., then slow rise 1 mm.*
13	—	·00075	15	—	Twitches, gradually increase then decrease. (Fig. 14.)
14	—	·00075	30	—	Rise 1 mm.
15	—	·00075	30	0	0*
16 & 17	—	·001	15	0	0
18	—	·001	30	0	0
19	18° C.	·001	15	—	0
20	18·5° C.	·001	15	—	1 No fall in 3 mins. Frog 32 grams.
21	19° C.	·001	15	—	0 Rise of 11 mm. in ¼ an hr.
22	19° C.	·001	15	—	·1 Rise in first 5 secs. (no fall), slowly passing into rigor rise. (Fig. 11.)
23 & 24	20° C.	·001	15	—	0 (Fig. 15 a.)
25	22° C.	·001	15	nic. ·03 1	— Fall below base line in 2¾ mins.†
26	22° C.	·001	60	½	— Fall below base line in 4 mins.†
27	22° C.	·001	15	0	0 Curari and nicotine solutions at 22° C.
28	22° C.	·001	15	nic. ·075 0	
29	22° C.	·001	22½ hrs.	0	Muscle shrunken, apparently dead, slowly stretches for several hours.

* Slight twitch on running off the nicotine.

† The lever was falling before adding nicotine. At high temperatures the muscle slowly stretches with a light weight and it may take half-an-hour or more before a constant length is attained. (Cp. Fig. 1.)

The experiments show that nicotine ·01 to ·1 p.c. has commonly no effect after the muscle has soaked in ·001 p.c. curari for 15 minutes, and when there is an effect, it is reduced to minimal proportions. Indeed since the slight occasional effect occurred when the temperature was abnormally high for frog's muscle, and the contraction reached its maximum in 5 to 10 secs. (cp. Fig. 11), it is probable that the rise was due to an action in the general muscle substance. One might expect

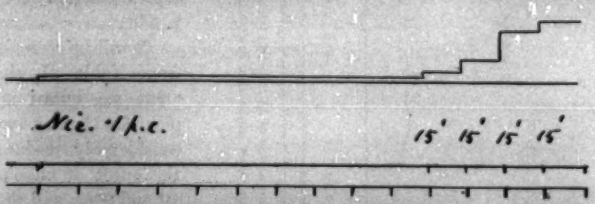

Fig. 11. × ½. Sartorius in ·001 p.c. 15 mins., then in nicotine ·1 p.c. Time in 10 secs. The steps in the tracing occurred in intervals of 15 mins. each.

that if the unstretched muscle were placed in ·1 p.c. nicotine, contraction, too weak to be marked in the graphic method, would be visible to the eye. I have made a few observations by this method. The sartorius was placed in ·001 p.c. curari for 15 mins., rinsed in Ringer's fluid, and then placed in 20 c.c. of ·1 p.c. nicotine. The results were much as by the graphic method. In warm weather (19° to 22° C.), there was no visible effect in four experiments, in cold weather in one out of four cases there was slight brief twitching.

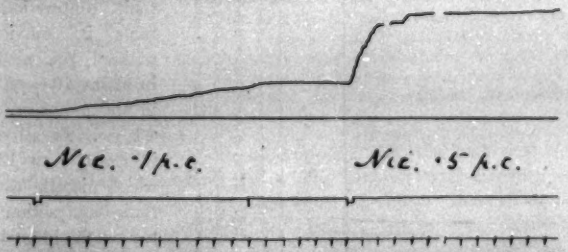

Fig. 12. Sartorius in curari ·0001 p.c. 15 mins., then in nicotine ·1 p.c. and ·5 p.c. The curve shows a slow, gradual rise with ·1 p.c. nicotine. Load 2½ grams. Time in 10 secs.

I have also made observations under a low power of the microscope of the effect of flooding the muscle with ·1 p.c. nicotine after a stay of 15 minutes in ·001 p.c. curari. Usually there was no change, but once or twice (in winter) the fibres became slightly crinkled in places, indicating slight local contraction (cp. Part I, p. 358).

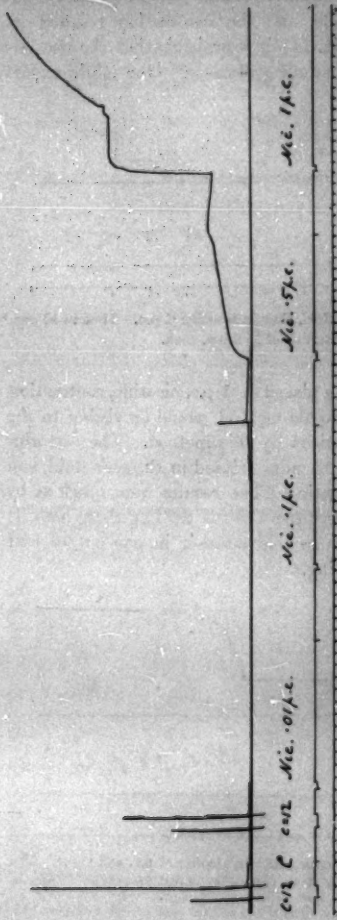

Fig. 13. ×½. Effect of successive solutions in nicotine of increasing percentage after a stay of 15 mins. in curari ·00075 p.c. The muscle was stimulated with electrodes at the two ends of the muscle before and after immersion in curari; the immersion occurred at *C*. Time in 10 secs. Load 1½ grams.

It will be seen also from Table II that ·0001 p.c. curari applied for 15 mins. very considerably reduces the effect of nicotine ·01 and ·1 p.c. and that a less percentage of curari is required to stop ·01 than to stop ·1 p.c. from causing contraction. It is certain then that a much smaller percentage of curari than ·0001 will stop the effect of ·001 p.c. nicotine. In the normal muscle, the tonic contraction attains its maximum with ·1 p.c. nicotine in about 20 secs. and in about 30 secs. with ·01 p.c. In all cases in Exps. 1 to 13 in which nicotine caused tonic contraction not too much obscured by twitches, the maximum height was more slowly attained than

normal. Fig. 12 (Exp. 1 of Table II) gives the best example of this. Normally after the muscle has been for 2 mins. (and probably much less) in ·01 p.c. nicotine, ·1 p.c. has no effect. In the curarised muscle at a certain stage (Exps. 2, 3, 8, 12) in similar circumstances a slight slow contraction is caused by ·1 p.c. nicotine (Fig. 13). Lastly in the few cases in which marked twitches were caused after curari by ·1 p.c. nicotine, the twitches instead of being as normal greatest at the beginning of the curve, were small at first and gradually increased (Fig. 14). All three facts afford proof that the nicotine gradually replaces the curari.

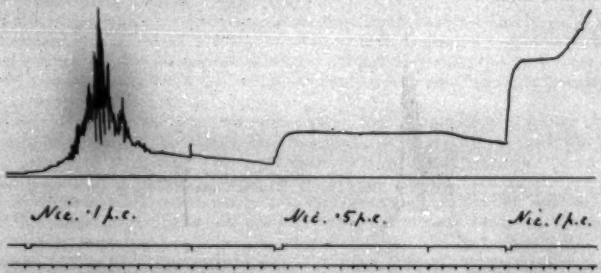

Fig. 14. ×½. Sartorius 15 mins. in ·00075 p.c. curari. The figure shows the strong twitches occasionally obtained with dilute nicotine and their gradual increase and decrease. Time in 10 secs. Load 1½ grams.

The occurrence of a displacement of curari by nicotine was tested in another way. The sartorius of a frog was placed in ·001 p.c. curari for 15 mins., in Ringer's fluid containing nicotine ·01 p.c. for 30 mins., and then a tracing taken of the effect of ·1 p.c. nicotine, a slight rise (1 mm.) followed by a like fall was obtained. Now we have seen that ·01 p.c. nicotine applied to a fresh muscle for about 2 mins. prevents ·1 p.c. nicotine from having an effect; I take it then that in the experiment given above the ·01 p.c. nicotine combined with the receptive substance as it was dissociated by the Ringer's fluid (cp. p. 262), and that the contraction obtained with ·1 p.c. nicotine was due to its displacing curari from its combination.

The experiments give some evidence that the paralysis of the receptive substance causing twitching, and of that causing tonic contraction, takes place at slightly different times. In most of them, when nicotine had any effect, it caused a trifling tonic contraction without any indication of twitching, in some there were slight twitches, in one (Exp. 13 and Fig. 14) there were many and strong twitches, so that the degree of tone could not be judged. In two other experiments

a similar tracing was obtained. These were incidental experiments. I wished to determine whether a ·2 p.c. solution of curari made up 9 days previously had lost any of its strength. It had been kept in a test tube plugged with cotton wool. From this a ·001 solution was made (*a*), and also a ·001 p.c. solution from the stock 1 p.c. solution (*b*). Two frogs were taken and a sartorius muscle from each placed in each of the curari solutions and after 15 mins. a graphic record was taken of the effect of ·1 p.c. nicotine. The nicotine had no effect on the muscles placed in (*b*), these are Expa. 23 and 24 of Table II. Both muscles placed in (*a*) gave rapid twitches, in one case lasting 50 secs. and in the other about 90 secs. and when the twitches were over there was very little tone. The

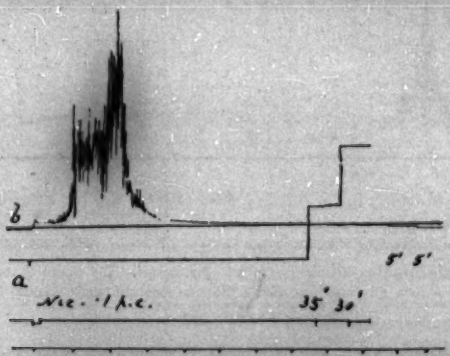

Fig. 15. ×⅔. (*a*) Right sartorius in curari ·001 p.c. 15 mins. (*b*) Left sartorius in curari of slightly less percentage. Both treated with ·1 p.c. nicotine. Load 1½ grams. Time in ½ mins.

more marked case is given in Fig. 15. In this connection I may recall that in the muscles of torpid frogs, and in muscles observed some time after death, punctiform application of ·1 p.c. nicotine frequently gives tone without twitching (op. Part I, p. 355).

In the preceding experiments the muscle was only left in each nicotine solution for 2 mins. On the theory that nicotine displaces curari some action might be expected if a longer time were allowed. Since however as we have seen (p. 240) the height of the contraction depends upon the rate of action of nicotine on the receptive substance a sufficiently slow substitution of nicotine for curari would cause no contraction detectible by the graphic method. For such an experiment

the flexor carpi radialis and the rectus abdominis muscles are better adapted. By the graphic method, when no contraction is caused in a curarised sartorius by nicotine in the first 15 secs., I have not obtained a rise followed by a fall in longer stay in nicotine. In time, depending upon the percentage of nicotine, a rise occurs, but in the cases in which I have continued the tracing still longer, it was clear that the rise was the beginning of the rigor rise (cp. Fig. 15 *a*). The result tends to show that if nicotine ·1 p.c. does not in a deeply curarized muscle when the curari is not washed out, affect the receptive substance in 2 minutes to the degree necessary to cause a rise in the tracing it will not do so in a detectible manner in any time.

I have made also a few experiments in which the muscle was placed in fluid and so free to contract. As said above (p. 247) a muscle placed in ·1 p.c. nicotine straightens out in a few minutes, and after an interval the bending or folding which passes into rigor comes on. In the experiments following the bending or folding came on much earlier in the curarised or partly curarised muscle.

Exp. Temp. 22° C. Four frogs were taken. The left sartorii were put in ·1 p.c. nicotine as controls. The right sartorii were treated as follows: (1) in curari ·001 p.c. 15 mins., then in nicotine ·1 p.c.; (2) curari ·0001 p.c.+·1 p.c. nicotine; (3) curari ·001 p.c.+·1 p.c. nicotine; (4) curari ·01 p.c.+·1 p.c. nicotine. The changes of form began earlier and progressed faster up to 1½ hours in (2) to (4) than in their controls. In (2) the change of form began rather later (40 mins.) and in another 10 mins. the muscle was nearly doubled up, whilst the control was still flat. The later changes were not observed, but in 6 hours they all had assumed the rigor form. I have not however found this result to be constant (cp. Table VIII, p. 268).

The short summary of the experiments given in Table II shows little difference in the effect of curari whether the muscle was left in it for 15 or for 30 minutes, but an examination of the curves seemed to show

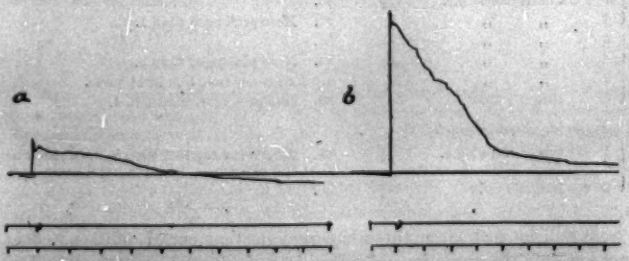

Fig. 16. ×½. Right sartorius in 25 c.c. ·00001 p.c. curari 24 hours, then in nicotine ·1 (*a*). Left sartorius in 25 c.c. Ringer's fluid for 24 hours, then in nicotine ·1 (*b*). Load 1½ grams. Time in ½ mins.

that the effect was rather greater in the latter than in the former case. I have accordingly tried the effect of a more prolonged stay in the diluter solutions.

TABLE III.

Effect on nicotine contraction of prolonging the immersion of the sartorius in curari.

Load 1½ grams. 20 c.c. of each solution was used except in *b* 2.

Series *a*. Room temp. 20° C.

Exp.	Solution	Time in solution in hours	Rise with ·1 p.c. nicotine.	Remarks
1	Curari ·0001 p.c.	½	6 mm.	Slight twitches, in 1½ mins. fall below base line.
2	,, ,,	2	0.	In 3 mins. fall below base line.
3	,, ,,	4	1½ mm.	Fall below base line in 2½ mins.
4	Ringer	4½	31 mm.	Not reach base line in 5 mins.
5	Curari ·00001 p.c.	6	0.	Slow fall.
6	,, ,,	24	0.	Fall, rather more than in 5.

The fall below the base line was going on before immersing the muscle in nicotine.

Series *b*. Temp. 19° C.

1	Curari ·0001 p.c.	6	28 mm.	Slight twitch at beginning.
2	½ c. nicotine ·0001 p.c.	24	10 mm.	Slow rise, slight twitches only.
3	Curari ·0001 p.c.	24	5 mm.	Primary twitch to 8 mm. falls below base line in 2 mins.
4	Ringer	24	35 mm.	Primary twitch, more rapid fall than usual.

Both curari and nicotine may have been a trifle less than the nominal strength as they were not made up from the stock 1 p.c. solution.

Series *c*. Room temp. 22° C.

1	Curari ·00001 p.c.	½	33.	
2	,, ,,	2	35.	More prolonged than 1.
3	,, ,,	4	36.	
4	,, ,,	6	31.	More prolonged than 3.
5	,, ,,	24	4.	Fall below base line in 1½ mins.
6	Ringer's fluid	24	20.	Less prolonged than 1 to 4.

Series *d*. Room temp. 22° C.

1	Curari ·00001 p.c.	½	35.	More twitching in 1 than in 2; contracture at end of 5 mins., 4 mm. in 1 and 5 mm. in 2.
2	Ringer's fluid	½	39.	

It will be seen from these experiments that curari ·0001 p.c. does not paralyse the receptive substance in a quarter of an hour, but does so in 2 hours, and that ·00001 p.c. curari does not completely paralyse the receptive substance in 24 hours (Fig. 16). The differences shown

in the early stages with ·00001 p.c. curari are probably due to differences in the muscles of the two sides.

It appears then that the rate of paralysis of the receptive substance decreases very rapidly with decrease in the percentage of curari, as we have seen above (p. 246) is also the case with nicotine.

2. *Duration of the curari effect.*

In the preceding experiments the curarised muscle was left a short time only in Ringer's fluid. Some experiments were also made to see whether the effect of curari would disappear after a longer stay in the salt solution. Muscles were placed for 15 mins. in curari, rinsed in Ringer's fluid, and each then placed in 30 c.c. of the fluid, and left in it for ½ to 24 hours before being tested with ·1 p.c. nicotine. (Table IV.)

TABLE IV.

Duration of curari paralysis.

(Load 1¼ grams.)

Series *a*.	Percentage of curari	Time in curari in mins.	Time in Ringer's fluid in hours	Effect of nicotine ·1 p.c.
1	·001	15	½	Slight twitch on immersion, with contracture; no further effect in 5 mins.
2	·001	15	½	Slow rise, ·5 mm.
3	·001	15	½	Considerable twitching, lasting 1½ mins. and some tone, the effect was much greater than in Fig. 17.
4	·01	15	½	Twitch 1 mm. on immersion, after 1 min. slow rise to 1·5 mm.
5	·01	15	½	0. (Slight twitch on running off nicotine.)
6	·001	15	½	Fig. 17 *a*.
7	·001	15	2	Fig. 17 *b*.
8	·001	15	6½	Fig. 17 *c*.
9	·001	15	24	Fig. 17 *d*.

Series *b*. Four sartorii were placed in ·001 p.c. nicotine for 15 mins., then in Ringer's fluid for 1, 2, 3 and 4 hrs. respectively, and then in nicotine ·1 p.c. In all there were some twitches and bending up of the muscles on placing in nicotine, but no certain difference in degree in the several muscles.

There is then a decrease of the curari paralysis when the muscle is left in Ringer's fluid (Fig. 17)[1], the decrease is more rapid at first, and then progresses very slowly so that even at the end of a day, the paralysing effect is still considerable.

[1] The return of twitching has varied much more than that of tone so far as the latter could be judged.

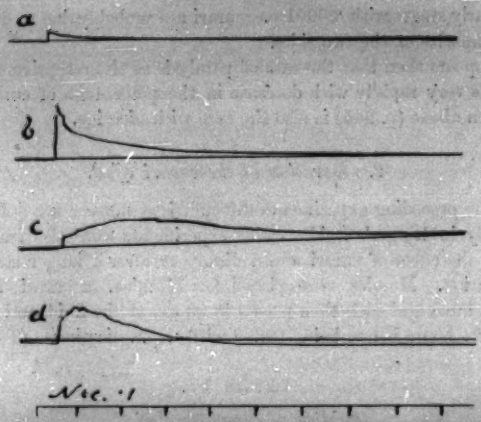

Fig. 17. 4 sartorii in curari ·001 p.c. 15 mins., then in Ringer's fluid (*a*) ½ hr., (*b*) 2 hrs., (*c*) 6½ hrs., (*d*) 24 hrs. The tracings show the contraction with nicotine ·1 p.c. (cp. Table IV. Exps. 6—9). Load 1½ grams. Time in ½ mins.

We have seen above that the effect of curari increases from ·00001 p.c. up to ·001, and that after the latter, ·1 p.c. nicotine does not cause contraction. The increasing effect might be due to more and more complete saturation, or (on the theory that the combination formed with the receptive substance depends upon the relative concentration of curari and nicotine) to the concentration of the curari in the muscle spaces being greater the greater the percentage of curari. In order to throw some light on this point, pairs of sartorius muscles were placed in curari solutions of different percentage, for the same time, left in Ringer's fluid for the same time, and then the effect of ·1 p.c. nicotine noted.

TABLE V.

Duration of curari paralysis with varying percentages.

Series *a*. Fig. 18. Pairs of sartorii were placed for 15 mins. in curari as follows:

1	·0005 p.c.	3	·001 p.c.	5	·001 p.c.	7	·01 p.c.
2	·01 ,,	4	·01 ,,	6	·1 ,,	8	1·00 ,,

Each was then placed in Ringer's fluid for an hour, and then a graphic record taken of the effect of ·1 p.c. nicotine; 1 gave a normal curve except that the twitching was rather greater, 2 and 5 gave contractions of the same type, viz. twitches lasting 20 to 30 secs.

followed by a slight tonic rise. The tonic rise was very slight in 3, but the first twitch was stronger than in 5.

2, 4 and 7 gave curves of the same type, viz. a rise accompanied by twitches lasting 20 to 30 secs., without any subsequent tonic rise (7, not given in Fig. 18, was like 4 but rather less), 6 and 8 gave a mere trace of rise, barely perceptible in 6.

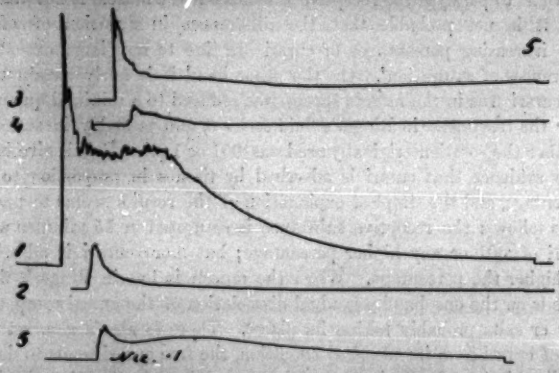

Fig. 18. × ½. Curarised muscles washed out with Ringer's fluid; then a tracing taken of the effect of ·1 p.c. nicotine (see *a*, Table V). The tracing was taken for 5 mins. and then the drum stopped for 5 mins.

Series *b*. Fig. 19. The sartorii were placed for 15 mins. in curari as follows:

1	·001 p.c.		3	·1 p.c.	
2	·01	,,	4	1·0	,,

Then rinsed in Ringer's fluid, and placed in Ringer's fluid (renewed at the end of each hour) in a shaking machine, 1 for 4½ hrs., 2 for 5 hrs., 3 for 5 hrs., 4 for 5½ hrs. A tracing was then taken of the effect of ·1 p.c. nicotine. The result in 1 and 2 is shown in Fig. 19. It will be noticed that in this case a greater contraction was obtained from the muscle treated with ·01 p.c. curari, this was a ½ hr. longer in the Ringer's fluid, but the result indicates that prolonged washing out tends to equalize the condition of the muscles. In 3 there was a very slight rise, soon over. In 4 there was no effect.

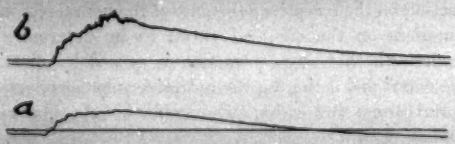

Fig. 19. Right and left sartorius muscles (*a*) 15 mins. in curari ·001 p.c.; and 4½ hrs. in Ringer's fluid, (*b*) 15 mins. in ·01 p.c. and 5 hours in Ringer's fluid. Effect of nicotine ·1 p.c. The tracing occupied 5 mins.

The experiments show that the duration of the effect increases with the percentage of curari from ·0005 up to 1 p.c. In series *a* the maximum effect was obtained with ·1, but in series *b* a difference between 1 and ·1 p.c. was perceptible. In view of the fact that ·001 p.c. is sufficient to paralyse the receptive substance if the muscle is not washed out, it is not probable that the differences in duration of effect with increasing percentages of curari are due to any large extent to differences of saturation[1]. On the other hand it is fairly certain that the curari free in the muscle spaces was reduced to a minimal quantity after the treatment in Ringer's fluid series *b*, and probably in series *a*, whether the solution originally used was ·001 or 1 p.c. I shall give later some evidence that curari is adsorbed by tissues in proportion to its percentage, and the simplest explanation of the results seems to me to be as follows: the receptive substance is saturated in 15 minutes with curari of ·001 or any higher percentage; but more curari is adsorbed the higher the percentage. When the muscle is left in Ringer's fluid there is on the one hand a gradual dissociation of the curari compound (ions or salts probably taking its place). There is also a slow setting free of curari from its adsorbed condition, the free curari tends to delay the dissociation of curari compound. On adding nicotine the adsorbed curari is set free at a faster rate (nicotine taking its place) and this cuts short the action of nicotine on the receptive substance.

On this view the differences would diminish after more prolonged washing out of the curari, and that this is the case is indicated by ser. *b*, Exps. 1, 2 in Table V (Fig. 19). I propose to make further experiments on the matter.

3. *Effect of curari on the action of* ·5 *to* 1 *p.c. nicotine.*

Solutions of nicotine ·25 to 1 p.c. cause as I have said an immediate shortening of the muscle by acting on the general muscle substance as well as by acting on the receptive substance of the neural region. There are two questions on the effect of curari to be determined in this connection, first whether any contraction is caused by nicotine antagonizing the curari and acting on the receptive substance, and secondly whether curari alters the action of nicotine on the general muscle substance.

[1] The determination of varying degrees of saturation presents difficulties, and I shall return to it in a later paper.

TABLE VI.

Effect of ·5 p.c. nicotine on the curarised sartorius.

The muscle was stimulated with a make and a break induction shock before the immersion in nicotine. The load was 2·5 grams. The height of the secondary rise, unless otherwise mentioned, was measured from the lowest point of the curve after the primary rise to the point attained at the end of 20 minutes from giving nicotine.

Series *a*. Dec.

Exp.	Percentage of curari	Time in curari in mins.	Primary rise in mm.	Extent of subsequent fall	Height of secondary rise	
1	·001	15	9·5	0	42·5	
2	·001	15	10	0	36	
3	·01	15	10·5	·5	39	
4	·1	15	9·5	1	22·5	
5	1·0	15	12	1	45	
6	1·0	120	37·5	0	37·5[1]	
7	1·0	120	32	3	7[2]	
8	1·0	120	40	14	11[3]	Fig. 21.

[1] Rise taken after 15 mins., slight fall in next 5 mins. then no rise or fall for 10 mins. [2] Rise taken after 10 mins. [3] Rise after 15 mins.

Series *b*. March. Some of the muscles were placed in Ringer's fluid instead of curari, this is indicated by 'Ringer' in column 2. In all cases the time of stay in the fluid was 15 mins. Load 2½ grams.

Exp.	Percentage of curari	Primary rise in mm.	Fall in mm.	Secondary rise	Total rise	
1	Ringer	24·5	16	16·5	25	Fig. 20 *a*.
2	·0001	11	5	25·5	31·5	Fig. 20 *b*.
3	Ringer	22	10·5	70·5	82	
4	·0001	14	1	61	74	
5	Ringer	38	28	58	68	
6	·001	9·5	0	53	62·5	
7	Ringer	27	19·5	36·5	44	
8	·001	4	0	29·5	33·5	
9	·01	6·5	0	45·5	52	
10	·1	8·5	0	45	53·5	
11	·01	6·5	0	70·5	77	
12	·1	8·5	0	70·5	79	
13	Ringer	32	15·5	74·5	91	

It is clear from these experiments that ·0001 p.c. curari greatly reduces the primary contraction caused by ·5 p.c. nicotine, *i.e.* if any immediate antagonism occurs it is only slight. Exp. 1 gives some indication that there is a slight slow antagonism since there is a greater secondary rise after curari than after Ringer's fluid, but no stress can be placed on a single experiment. On comparing the effect of ·0001 p.c.

and ·001 p.c. curari it appears that the latter has a somewhat greater effect in reducing the nicotine contraction; this may reasonably be attributed to the paralysis of the receptive substance by ·001 p.c. curari being more complete. Allowing for this, a very small part of the rise can be due to an antagonising action; and as the secondary rise is less after ·001 p.c. than after Ringer's fluid there is no evidence of a slow antagonising action. I conclude then that when the receptive substance

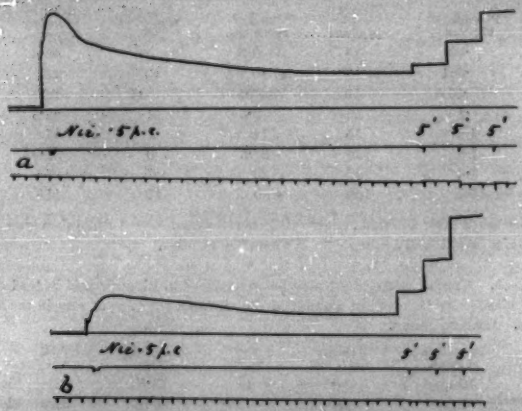

Fig. 20. ×¾. Effect of nicotine ·5 p.c. on (a) right sartorius placed in Ringer's fluid, and on (b) left sartorius placed in curari ·0001 p.c. for 15 mins. (cp. Table VI b, Exps. 1 and 2). Load 2½ grams. Time in 10 secs.

has been paralysed as regards ·1 p.c. nicotine, ·5 p.c. nicotine has extremely little if any antagonising action on the curari paralysis in the time taken by the primary contraction. The experiments give no evidence in this case of a slow substitution of curari by nicotine but it is not likely that if this occurred it would be appreciable.

The effect of ·01 and ·1 p.c. curari it will be convenient to take after that of 1 p.c. It will be seen from Table VI series *a* that the sartorius after being placed in 1 p.c. curari for 15 minutes gave an appreciably higher primary rise with ·5 p.c. nicotine than when curari ·001 to ·1 p.c. had been used, and that the rise very greatly increased when the muscle remained in the solution for 2 hours (Fig. 21). In all these experiments on prolonged curari action the irritability to induction shocks was very

slight. The secondary rise was decreased but had a different form in each experiment. Obviously the prolonged stay in the curari had injured the muscle; the effect however may be due to the salts of curari and not to curarine.

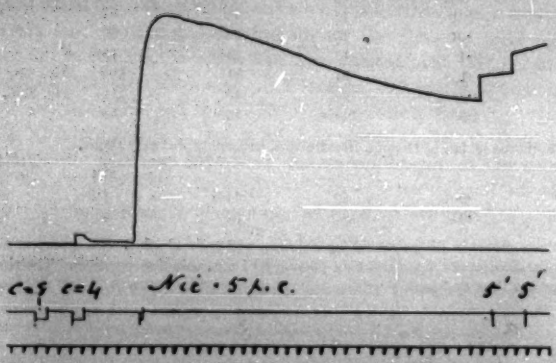

Fig. 21. Effect of ·5 p.c. nicotine on sartorius which had been placed in 1 p.c. curari for 2 hours. Load 2½ grams. Time in 10 secs. (cp. Table VI *a*, Exp. 8).

Taking now the effect of solutions of curari neither very dilute nor very strong, we see that in ser. *a* there was no appreciable difference between the effect of ·001 and of ·1 p.c. Allowing for the fact that the muscles were taken from different frogs, it is still obvious that curari up to ·1 p.c. has in 15 minutes very little effect on the general muscle substance. In ser. *b* there is a slight difference in the primary rise after ·01 and ·1 p.c. nicotine in both of the experiments made. The difference in this case is I think genuine and not due either to differences in the muscles or in the mode of handling them. In that case it must either be due to nicotine antagonising the curari slightly after ·01 curari and not after ·1, or to ·1 p.c. curari slightly increasing the nicotine effect, as we have seen stronger solutions do. I take it to be due to the latter. For when the muscle is left 2 to 3 hours in ·005 to ·1 p.c. nicotine, or in 1 p.c. for a shorter period, ·1 p.c. nicotine generally causes an immediate rise in the tracing and this in some cases passes without a plateau into rigor.

I have made a few similar experiments with 1 p.c. curari.

TABLE VII.

Effect of curari on the contraction caused by 1 p.c. nicotine.

Exp.	Percentage of curari	Time in curari in mins.	Primary rise in mm.	Total rise in 15 mins.	
1	·001	15	30·5	92	
2	·01	15	19	87	
3	·01	15	29	83	
4	·1	15	31·5	73·5	
5	·1	15	33	110	Fig. 22 *a*
6	·5	15	40	111	Fig. 22 *b*.
7	1·0	120	45 [1]	46	

[1] Followed by fall of 11 mm., the electrical irritability was very slight.

				Fall	Total rise in 5 mins.
8 [2]	·001	30	41·5	4·5	85
9	·01	30	37	3	85

[2] 8 was placed in Ringer's fluid for 30 mins., and 9 for 40 mins. They were immersed in ·1 p.c. nicotine for 2 mins. before putting in 1 p.c., this had no effect. The rise in 8 was quicker than that in 9.

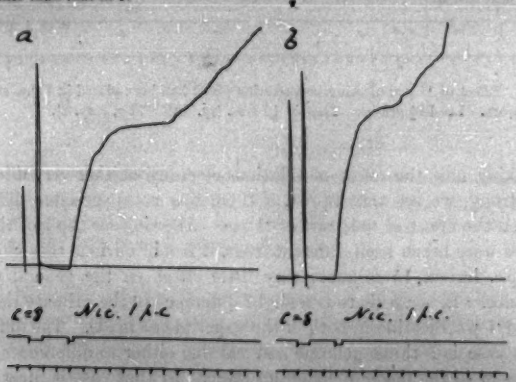

Fig. 22. ×$\frac{2}{3}$. Effect of ·1 p.c. nicotine on (*a*) right sartorius placed in curari ·1 p.c. for 15 mins. and on (*b*) left sartorius placed in curari ·5 p.c. for 15 mins. (cp. Table VII, Exps. 5 and 6). Load 2$\frac{1}{2}$ grams.

The general results it will be seen are similar to those with ·5 p.c. nicotine. Curari above a certain percentage—apparently beginning about ·1 p.c.—increases the primary rise caused by 1 p.c. nicotine; and it causes in proportion to the concentration and to the duration of action a decrease in the secondary rise and a great reduction in irritability to

electrical stimuli. In Exps. 1 and 2 the primary nicotine rise was much greater after ·001 p.c. than after ·01 p.c. curari, but as the rise after ·01 p.c. was less than usual, the difference may have been more or less due to a difference in the muscles. In Exps. 8 and 9 the muscles were cut out of the body within a few minutes of one another and placed in Ringer's fluid for about half an hour. Nicotine 1 p.c. caused a distinctly quicker and a somewhat higher rise followed by a rather greater fall in the muscle which had been treated with ·001 p.c. curari. The results indicate that there is a partial immediate antagonising of the action of ·001 p.c. curari by 1 p.c. nicotine.

The primary nicotine rise is slightly greater after ·1 than after ·01 p.c. curari: this may have been due to a difference in the muscles of the two sides, or, as I have suggested above in speaking of the effect of ·5 p.c. nicotine, mark the beginning action of curari upon the general muscle substance.

In a few experiments in which ·25 p.c. curari was used, curari was also added to the successive nicotine solutions to make ·25 p.c.; the contraction with 1 p.c. nicotine was not appreciably altered.

In my preliminary account I stated that ·5 to 1 p.c. nicotine caused a greater primary rise after curari than after dilute nicotine and I attributed this to the contraction in the neural region of the muscle caused by dilute nicotine lowering the irritability in this region. The conclusion was based upon the results of a large number of experiments, but they were made for the most part on different frogs. In some subsequent experiments in which the right and left sartorius of the same frog have been compared, I have not obtained constant results and further experiments on the point are required.

4. *Effect of curari after nicotine.*

I have shown earlier that in the coraco-radialis and flexor carpi radialis muscles[1], curari applied a few minutes after dilute nicotine causes relaxation or increases the rate of that going on. Since then I have experimented both on these and on other muscles in the frog. In all cases when curari is applied soon enough after dilute nicotine, there is a decrease in the effect of the nicotine; shown by a more rapid rate of relaxation, and in certain conditions by a decrease in the height of contraction. When curari is applied late during the residual contraction there is little or no effect.

[1] Tracings of these effects were shown at the Intern. Physiol. Congress in 1907.

The time after immersion in nicotine at which curari increases the rate of relaxation varies widely in different muscles; it depends also upon the percentage of nicotine and the duration of the contraction. In the sartorius after ·1 p.c. nicotine, curari applied even for as short a time as 20 secs. after the nicotine may have no effect in hastening the rate of relaxation. The flexor carpi radialis and the rectus abdominis muscles have a wider range of response to nicotine, and the contraction is higher and more prolonged; in these muscles some relaxation is obtained with curari for more than ½ of an hour after immersion in nicotine. I reserve a detailed consideration of the effects till a later time; but the general conclusions to be drawn from the experiments are I think clear. These are, first that curari in all cases puts an end to the stimulating action of nicotine, and secondly that it has little and perhaps no effect upon the state which is caused by the stimulation. In other words curari paralyses the receptive substance but (in the percentage given in these experiments) has little or no action on the general muscle substance whether this is at rest or contracted (cp. also p. 290).

5. *Effect of curari on the survival irritability in dilute nicotine.*

In a few experiments made in winter, the irritability of curarised sartorii lasted longer in ·01 to ·1 p.c. nicotine than that of the uncurarised muscles. I have made further experiments on the point. There were two exceptions out of seven cases, so that the possibility that the difference found in survival irritability is simply due to differences in the muscles in the two sides, is not excluded. The later experiments were as follows:

TABLE VIII.

Series *a*. Temp. 13° to 11° C.

1	Curari ·001 p.c. 15′, then nicotine ·1 p.c.	
2	Ringer's fluid 15′	,, ,, ,,
3	Curari ·01 p.c. 15′	,, ,, ,,
4	Ringer's fluid 15′	,, ,, ,,
5	Nicotine ·1 p.c.	
6	Curari ·001 p.c. + nicotine ·1 p.c.	

5 *hours*. 1 and 2. Little change in either, but a trifle more in 2 (change is in length, in curve to lateral border, and in bend at knee end).
3 and 4. Fold in nerve region, and bent at the fold, 4 much more than 3.
5 and 6. Nearly normal shape, 6 a trifle shorter.

TABLE VIII (*cont.*).

21½ to 23½ *hours.* 2 is rather more shrunken than 1, has nearly assumed rigor form. 1 has a double bend and is more irritable than 2.

3 and 4 have both nearly complete rigor form; 3 only traces of irritability; 4 the same, except on lateral border where better irritability.

5 and 6 have both nearly complete rigor form; 5 has slight irritability on lateral border, 6 not irritable.

Series *b*. Room temp. 18° C.

1 Nicotine ·01 p.c. for ½ hour, then in nicotine ·1 p.c.
2 Curari ·002 p.c. ,, ,, ,, ,,

3 Nicotine ·1 p.c.
4 Curari ·001 p.c. + nicotine ·1 p.c.

5 Nicotine ·1 p.c. made up in NaCl ·7 p.c.
6 Ringer's fluid.

At the end of 19 hours, the condition was as follows:

1 Partly shrunken, much folded, irritability slight.
2 Flat, slightly bent at knee end, irritability much better than in (1).
3 Rigor form, but not opaque white; irritability trace only.
4 Partly shrunken, with some folds; irritability better than in (2).
5 Rigor form, more shrunken and more opaque than (3), irritability gone.
6 Flat, little if at all shrunken, irritability fair, distinctly better than in 4.

Series *c*. Room temp. 22° C.

1 Ringer's fluid 15′, then nicotine ·025.
2 Curari ·005 15′, then nicotine ·025.

3 Nicotine ·025.
4 Curari ·005 and nicotine ·025.

22 *hours.* 1 and 2 in rigor form, irritability slight, a trifle better in 2 than in 1.

3 and 4, folded and somewhat shrunken, irritability better than in 1 and 2, and better in 4 than in 3.

6. *The primary effect of mixtures of nicotine and curari.*

From the point of view of testing how far tissues take up nicotine after curari poisoning (cp. p. 284) it is important to know the degree of contraction, if any, which is caused in muscles by different mixtures of the two poisons. In order to determine this, mixtures containing different proportions of nicotine and curari were dropped over the muscles and a graphic record taken. I give here the experiments on the sartorius.

TABLE IX.

Series a. July. Load 1½ grams.

1	Curari	·0005	+ nicotine	·1.	The first three at the beginning gave a violent twitch of
2	,,	·002	+ ,,	·1.	7 or more cms., small twitches for a minute or two and
3	,,	·02	+ ,,	·1.	residual tone of 3 to 7 mm. at the end of 10 mins.
4	,,	·2	+ ,,	·1.	(4) gave slight twitch and tone (rise of 5 mm.), the lever reached the base line in 3 mins. and then fell slightly below it.

Series b. Aug. Load 1½ grams.

					Rise	
1	Curari	·0005	+ nicotine	·01.	2 mm., reaches base line in 4 mins.
2	,,	·002	+ ,,	·01.	2½ mm., 1½ above base line in 5 mins.
3	,,	·02	+ ,,	·01.	1½ mm., fall below base line in 3½ mins.
4	,,	·2	+ ,,	·01.	0, fall below base line in 3 mins.

Thus nicotine ·01 can be detected in the presence of curari up to about ·02 p.c., and nicotine ·1 p.c. in the presence of curari ·2 p.c. The arm and abdominal muscles as will be shown at a later time are much more delicate indicators.

7. *The effect of nicotine in the early stage of paralysis of nerve effect by curari.*

On the theory I have put forward the nerve impulse affects primarily the receptive substance. It has long been known[1] that the tonic action of nicotine in the arm muscles begins before the nerves are paralysed. For on injecting nicotine under the skin of a frog, the cataleptic condition of the arm muscles is obvious at a time when voluntary movement of the arms still occurs (cp. also Part II p. 203).

A more interesting point is the condition of the receptive substance as regards nicotine stimulation, when electrical stimulation by way of the nerves has no effect. This I have investigated in the following manner. The brain of a frog is destroyed and curari injected into the dorsal sac; when reflex action ceases, the nerve to the muscle to be observed is stimulated with strong interrupted currents. If this has no effect, nicotine is applied locally to the muscle or it is cut out, and either placed in nicotine ·01 or ·1 p.c. and the effect observed with the eye or a graphic record taken.

The paralysis of the nicotine twitchings is nearly synchronous with the paralysis of nerve effect but as a rule outlasts it a little. This I

[1] Cp. Langley and Dickinson. This *Journal*, I. p. 270. 1890.

have observed in the sub-maxillary muscle, the thigh muscles, the pectoral-abdominal, and the transverse and oblique abdominal muscles, but the twitchings were always much less than normal. In some cases, and more frequently in the thigh muscles than in the others, there were no twitchings in an early stage of paralysis of the nerve endings, but it is to be remembered that in some frogs the uncurarised thigh muscles show very little twitching. The time of paralysis of the receptive substance for tonic contraction is markedly different in different muscles. In the sartorius it outlasts very little the twitchings, i.e. in this muscle —and apparently in other thigh muscles—the paralysis of the nicotine tonic contraction is nearly synchronous with the paralysis of nerve effect. It usually lasts a little longer in the sub-maxillary muscle, and longer still in the digital muscles. The contraction of the digital muscles is easily seen by placing the leg, after removal of the skin, in 1 p.c. nicotine. After a dose of nicotine considerably larger than is necessary to paralyse the nerve effect, a graphic record of the flexor carpi radialis and of the rectus abdominis treated with ·01 p.c. nicotine shows a fair contraction in the former, and a good contraction in the latter. A similar result on the gastrocnemius is mentioned below (p. 281). After a large dose of curari and sufficient time of action none of the muscles give any contraction with nicotine.

Thus as regards the quick conducted contraction, the effect of nicotine outlasts the nerve effect but a short time, and probably only in fresh irritable muscles. It is clear that the nerve impulse is a much more effective stimulus than nicotine on the receptive substance for quick conducted contractions. As regards the receptive substance causing the tonic contraction we have no definite evidence that this can be stimulated by nerve impulses, and we have seen that in some muscles stimulation of the motor nerves with tetanising currents causes no contraction at a time when the receptive substance responds fairly readily to nicotine. It is possible then that the 'tonic' receptive substance is only put in action by some metabolic products carried to it in the blood, and this might help to account for the prolonged contraction of the arm muscles of the frog during the breeding season. But there are several facts, and especially the localization of the receptive substance in the region of the nerve endings, which make it unsafe at present to conclude that nervous impulses are without effect. We may however conclude that if the nerve impulses set up by tetanising currents do affect it, their paralysis by curari is but a stage in the progressive paralysis of the receptive substance.

IV. Effect of neutral salts on the nicotine contraction.

On this subject I have only made sufficient experiments to form some idea whether different salts have a different action on the receptive substance, on the primary rise caused by 1 p.c. nicotine and on the secondary (rigor) rise. The salts, the effect of which was tried, were the chlorides of Na, K, Ca and Mg. The usual method was as follows: the sartorius was fixed in the apparatus and stimulated with a make and a break induction shock giving a sub-maximal response, the currents passing through the muscle from end to end; the vessel containing the muscle was filled with the salt solution for a given time, as a rule 15 mins., the solution run off and the muscle again stimulated. Finally the nicotine solutions, as a rule successive solutions of increasing strength, were added. By the method used it is not likely that the induction shocks remained constant, but I have no doubt they were sufficiently constant to show any considerable variation in the condition of the muscle, moreover the results agree in the main with those of previous observers[1]. It was only desired to compare broadly the response of the muscle to induction shocks with that to nicotine.

NaCl. Sodium chloride was used in ·6 p.c. solution in distilled water and the muscle left in it for 15 mins. The response to induction shocks was much increased and usually much prolonged (cp. Figs. 23, 24). Running the solution in and out of the vessel constantly caused a twitch of the muscle of considerable height.

Ringer (This *Journal*, VIII. p. 292. 1887) noticed that after perfusion of a frog with ·6 p.c. NaCl solution the period of relaxation was enormously prolonged, and that there was sometimes great increase in the height of contraction. Locke (*Pflüger's Arch.* LIV. p. 501. 1893) found at certain seasons of the year that strong induction shocks passed through a sartorius which had been soaked for some time in NaCl ·6 p.c. caused a high tetaniform contraction lasting several seconds. The absence of effect when it occurred he was inclined to attribute to a higher temperature. In the Exp. mentioned below made in August, and in which all the fluids were at 22° C., the contraction obtained with a weak induction shock after treatment with NaCl ·6 p.c. was like that given in Fig. 23. Mines (This *Journal*, XXXVII. p. 408. 1908), passing the currents through a small portion of the muscle, finds that the irritability to induction shocks is not affected by treating the sartorius for a few minutes with NaCl ·7 p.c., but that after prolonged treatment the contraction was often much increased for the first stimulus.

On immersing the muscle in ·001 p.c. nicotine, there was a fairly strong contraction followed by a series of twitches (Fig. 23) lasting 2 or

[1] Observations on the effect of Na, K, and Ca salts in electrical irritability by a more accurate method have recently been made by Mines (This *Journal*, XXXVII. p. 408. 1908).

more minutes. Subsequent ·01 p.c. had a variable effect, once renewing the twitches, once having a slight effect (Fig. 23), once having no effect; the result apparently depended partly on the state of the muscle, and partly on the temperature. Nicotine ·1 after ·01 had no effect. When all the fluids were warmed to 22° C. (one Exp. only), the twitchings with ·001 p.c. were sooner over and subsequent ·01 had no effect. Nicotine 1 p.c. after the ·1 p.c. caused a contraction higher than normal except in

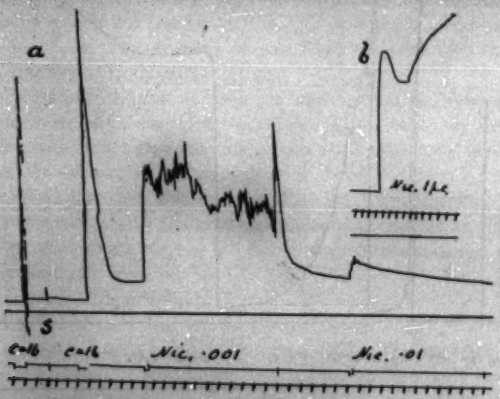

Fig. 23. ×⅔. Effect of NaCl on the nicotine contractions:

(*a*) Left sartorius. At *S* the muscle was placed in ·6 NaCl for 15 mins. Nicotine ·1 p.c. after the ·01 p.c. had no effect. Load 1½ grams. Time in 10 secs.

(*b*) Right sartorius. All fluids at 22° C. Effect of 1 p.c. nicotine after ·001, ·01, and ·1 p.c.

the experiment in which the fluids were warmed to 22° C. The effect in this case is given in Fig. 23 *b* as it shows a marked fall after the primary rise. On immersing the muscle in ·1 p.c. nicotine, there was a much larger first contraction, with numerous small twitches on a rapidly descending curve (Fig. 24). Subsequent 1 p.c. caused a contraction much higher than normal.

With ·5 p.c. nicotine the first part of the curve was like that with ·1 p.c., but the fall stopped about half way, and subsequent 1 p.c. gave a convex rigor rise, with slight depressions as from irregular shrinking.

With 1 p.c. nicotine there was a much larger rise, with small twitches on the fall; the curve was similar to that in Fig. 4 *a* but the top was less rounded.

The twitches with ·001 to ·1 p.c. nicotine did not occur when the muscle was placed for 15 minutes in ·01 p.c. curari before being placed in the salt solution[1], but the rise caused by 1 p.c. nicotine was greater than normal.

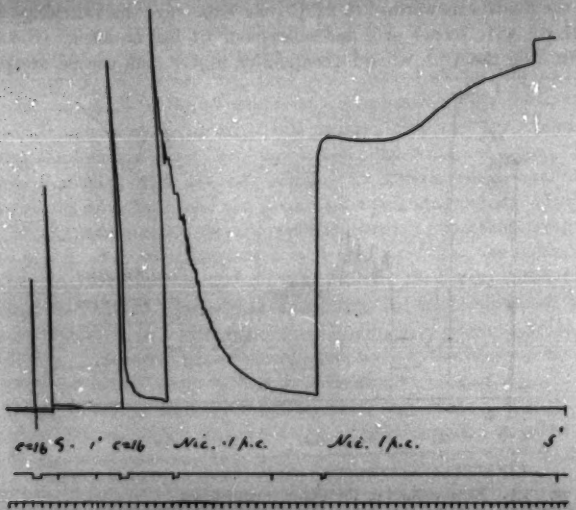

Fig. 24. ×¾. Sartorius immersed in NaCl ·6 p.c. 15 mins., at *S* the contraction occurred on running off the solution. Muscle stimulated with secondary coil at 16 cm. before and after immersion in NaCl. Immersed in ·1 p.c. and then in 1 p.c. nicotine. Load 1½ grams. Time in 10 secs.

[1] There may of course be twitchings in the NaCl solution. Ringer and others have shown that dilute curari does not stop the twitchings in NaCl solution. Mines (*op. cit.* p. 416) finds that ·2 p.c. curari prevents them from occurring, but that the salts contained in the curari act in the same way.

Previous treatment with dilute nicotine (15 to 30 minutes in ·1 p.c.) makes little difference to the twitchings caused by placing the sartorius in NaCl ·6 p.c. In some early experiments in which dilute nicotine was made up in NaCl ·6 instead of in Ringer's fluid, the twitchings soon stopped in nicotine ·1 to ·012—though contractions began on transferring to pure NaCl ·6 p.c. Since then I have made a few more experiments. On placing the muscle in nicotine ·01 p.c. in NaCl ·6, twitchings occurred though to a less extent than in the control; they could however be readily induced by moving the muscle about or by electrical excitation, and on excitation they occurred after 24 hours. When the muscle was placed in nicotine ·1 p.c. in NaCl ·6 p.c. the twitchings stopped in a few minutes. It may be noticed that the twitchings in NaCl ·6, except perhaps at first, are often local and not conducted throughout the fibres affected.

The most decided effect then of a short stay in sodium chloride is to increase the nicotine twitchings. The twitchings are due to an action in the receptive substance since they are readily stopped by curari. Whether the immersion in sodium chloride increases the irritability of the receptive substance or simply the response of the muscle the experiments do not definitely decide. The response of the general muscle substance to ·5 and 1 p.c. nicotine was it is true in nearly all cases distinctly higher (cp. Fig. 24) than is usual in similar circumstances when the muscle is not treated with sodium chloride, but no comparison was made of the two muscles of one frog, one with and the other without immersion in the salt solution.

The experiments give no evidence that sodium chloride increases the tonic contraction caused by nicotine. The curve may keep well above the base line, but when this occurs, it appears to be due to the repeated twitchings; when the twitchings cease, the curve is below the usual level rather than above it (cp. Fig. 24). This question I shall consider in dealing with the arm and abdominal muscles in which the twitchings are either absent or too feeble to affect the tracing.

The other salts were not used in pure solutions. A ·5 p.c. solution was made up in Ringer's fluid.

KCl. The muscle was left in the potassium chloride solution 15, 30, 60, and 90 minutes. It is known that KCl in this strength rapidly destroys the electrical irritability. Even after 15 minutes' stay in the

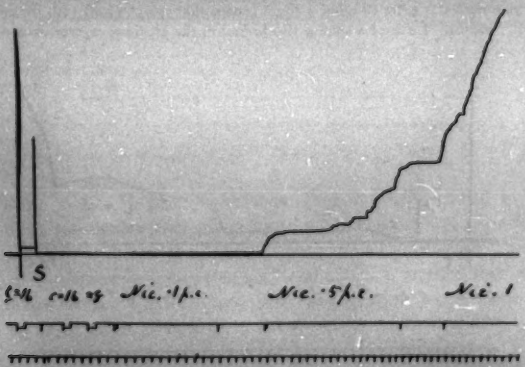

Fig. 25. ×⅔. Sartorius stimulated with secondary coil at 16 before immersion for 15 mins. in ·5 p.c. KCl in Ringer's fluid, and with secondary coil at 16 and at 8 afterwards. Nicotine ·1, ·5 and 1 p.c. Load 2½ grams. Time in 10 secs.

solution, induction shocks with the secondary coil at 8 cm. caused no contraction although a contraction of several centimetres had previously been obtained with the coil at 16 cm. (Fig. 25), ·1 p.c. nicotine had no effect, ·5 p.c. nicotine caused a slight rise, and 1 p.c. a greater one, irregular at first (cp. Fig. 25) then smooth and convex to the abscissa. The height of the rise in 15 mins. with 1 p.c. nicotine decreased with increased duration of the action of KCl.

Since muscle contains *K* salts it would be expected that a strong extract of muscle would paralyse the receptive substance and injure the irritability of the muscle. This was found to be the case.

Four grams of arm muscles were taken and ground with 4 c.c. of Ringer's fluid, boiled, a drop of acetic acid added when boiling, filtered, and a drop of Na_2CO_3 added to neutralize = (*a*) 1·5 c.c. the filtrate was dried, and burnt, and the ash ground up with 1·5 c.c. of distilled water = (*b*). In each fluid (*a*) and (*b*) a sartorius muscle was left for 15 min.; the one in (*b*) gave slight twitches. Each muscle was then placed in the apparatus. The irritability to strong induction shocks was very slight; nicotine ·1 p.c. caused no contraction, the contractions with ·5 p.c. and 1 p.c. nicotine were greatly reduced, the muscle treated with (*a*) gave with 1 p.c. nicotine a convex curve only and no primary rise.

$CaCl_2$. The effect of calcium chloride (·5 p.c. in Ringer's fluid) for 15, 60, and 110 minutes was tried.

After 15 mins. the irritability to induction shocks was reduced, but a contraction was still obtained with the secondary coil at 16 cm.; nicotine ·1 p.c. caused a rise of 1·5 mm. in the first 20 secs. and then no further rise or fall (Fig. 26); the rise with ·5 p.c. nicotine was fairly normal, that with 1 p.c. became markedly convex in a few minutes and was higher than normal. After 60 mins. a slight contraction was obtained with the secondary coil at 12; nicotine ·1 caused a similar rise to that in the previous experiment but only of

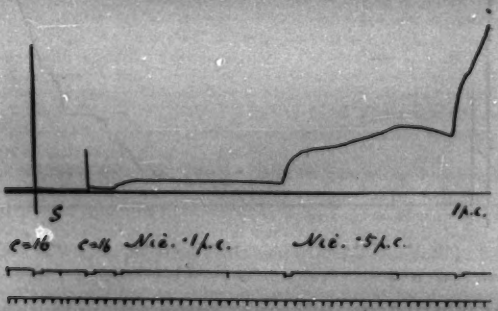

Fig. 26. ×½. Sartorius stimulated with secondary coil at 16, before and after 15 mins. in $CaCl_2$ ·5 p.c. in Ringer's fluid (at *S* in the tracing). Immersed in nicotine ·1, ·5 and 1 p.c. Load 2½ grams. Time in 10 secs.

·5 mm.; nicotine ·5 and 1 p.c. had much the same effect as before. After 100 mins. the irritability to induction shocks was further decreased; nicotine ·1 p.c. had no effect; ·5 and 1 p.c. had much less effect than in the other experiments.

These experiments show that excess of $CaCl_2$ in Ringer's fluid whilst reducing the electrical irritability of the general muscle substance reduces[1] also and in time abolishes the effect of the receptive substance on it. How far this is due to a direct action on the receptive substance is not easy to determine. The primary rise with ·5 and 1 p.c. nicotine was not increased, but the rigor rise with 1 p.c. appeared to begin earlier and reach a greater height.

Two experiments were made in which the muscle was placed in ·5 p.c. $CaCl_2$ in Ringer's fluid for 15 mins. and then transferred to a vessel containing ·01 p.c. nicotine made up in Ringer's fluid. In neither case were there twitchings or any obvious contraction. After 4 hours they were placed in NaCl ·6 p.c. and in time twitching and bending of the muscles commenced.

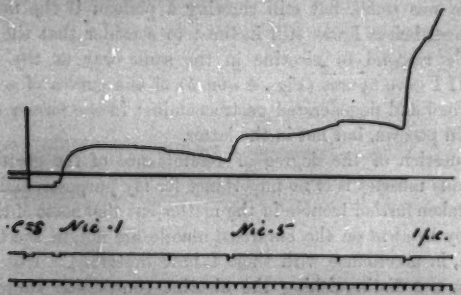

Fig. 27. ×⅔. Sartorius in $MgCl_2$ ·5 p.c. in Ringer's fluid, for 1 hour. Load 2½ grams.

$MgCl_2$ ·5 p.c. in Ringer's solution even in an hour had no great effect upon the contraction given by ·1 p.c. nicotine (Fig. 27) although it considerably reduced the irritability to induction shocks. The rise with 1 p.c. nicotine had, as after KCl and $CaCl_2$, a marked tendency to the convex form. In this case then the general muscle substance appeared to be more affected than the receptive substance.

[1] The form of the curve with ·1 p.c. nicotine (cp. Fig. 26) suggests an action on the general muscle substance, if that is so the receptive substance is paralysed early and the response of the general muscle substance is increased.

The Gastrocnemius.

The nicotine contraction of the gastrocnemius muscle and the effect of curari on it.

In the few experiments on the gastrocnemius referred to in Part I[1], p. 366, the contraction caused by dilute nicotine began slowly to fall soon after the maximum height of contraction had been obtained. In consequence I regarded the gastrocnemius as belonging to the same class as the sartorius in its response to nicotine. Boehm in his account of the effect of nicotine (and of other substances) on the gastrocnemius of *R. esc.* gives one curve (op. cit. Fig. 4) of a somewhat similar kind to those I had obtained, but in another figure (op. cit. Fig. 3) the rise is followed by a nearly straight line. In some later experiments (Part II[2], p. 211) I obtained a plateau to the curve with a light weight, but a curve slowly falling from the maximum with a heavier weight, if the muscle was small, but still showing a plateau if the muscle was large. Nevertheless I was still inclined to consider that the fibres of the muscle respond to nicotine in the same way as the sartorius. In Part III I gave figures (Figs. 4 and 5) of the curves of contraction of the normal and degenerated gastrocnemius; in the former there was a prolonged plateau, but not in the latter.

The question of the degree of resemblance of the gastrocnemius and sartorius muscles is of no importance for my purposes, and I should not have taken further trouble in the matter but that some of the results obtained by Boehm on the curarised muscle are not, in the form they are stated, in accordance with those I have described. This is chiefly due to his not distinguishing the nicotine contraction caused by an action in the receptive substance from that caused by an action on the general muscle substance.

The response of the gastrocnemius to nicotine decreases more rapidly when left in the body after death than does that of the sartorius, and this must always be taken into account (cp. p. 237 and Fig. 36).

Boehm did not find that the form of the curve was affected by the load. No doubt this was not intended to refer to considerable differences in load, but Boehm does not mention the limits he tried. In *Rana temp.* there is a marked difference in the curve with a load of 1½ and a load of 8 grams. In Fig. 28 it is seen that the maximum height is

[1] This *Journal*, xxxvi. 1907. [2] *Ibid.* xxxvii. 1908.

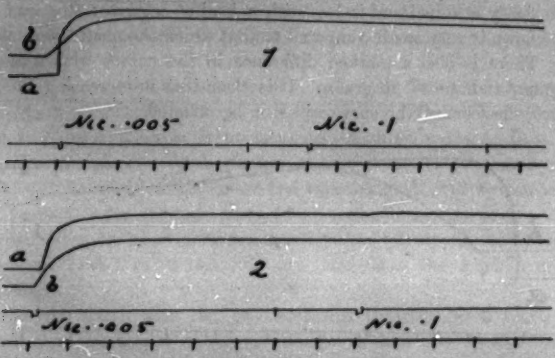

Fig. 28. × ⅔. 1. (*a*) Left gastrocnemius, load 1½ grams. (*b*) Right gastrocnemius, load 8 grams. Muscle (*a*) was cut out first.
2. A similar experiment but muscle (*b*) was cut out first.

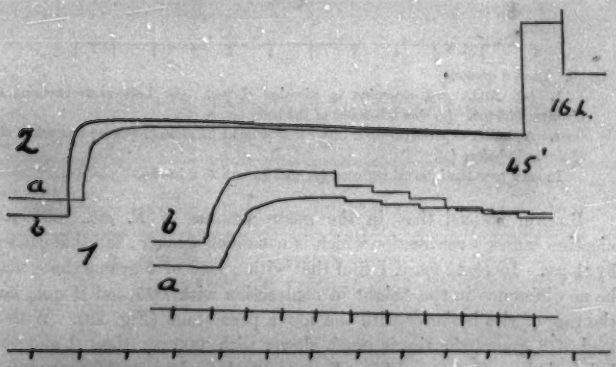

Fig. 29. Gastrocnemius. Load 5 grams. Time in ½ mins.
1. (*a*) Left muscle in nicotine ·005 p.c.
(*b*) Right muscle in nicotine ·1 p.c.
The steps in the curve mark intervals of 10 mins. each.
2. (*a*) Left muscle in nicotine ·1 p.c.
(*b*) Right muscle in nicotine 1 p.c.
In (*b*) the drum was stopped for 45 mins. and then for 16 hours.
Muscles (*b*) were taken out of the body after (*a*), so that the rise in (*b*) is somewhat less than normal.

more slowly attained and is less with a load of 8 grams; this load as is well known is very small compared to that which the gastrocnemius can lift. There is also a marked difference in the curves with a load of 10 grams and one of 20 grams. It is clear that in increasing the load from 0, the form of the curve will soon be affected.

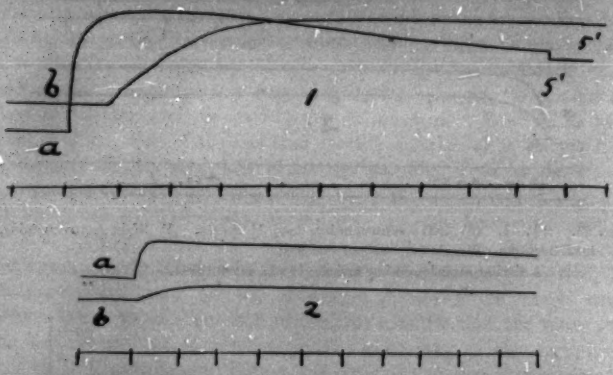

Fig. 30. Load 8 grams.

1. (*a*) Right gastrocnemius in nicotine ·1 p.c. (*b*) Left gastrocnemius in nicotine ·005 p.c. (*a*) was taken out of the body before (*b*).

2. A similar experiment on a small frog (weight 12 grams), but (*b*) was taken out of the body before (*a*).

In each case the interval between (*a*) and (*b*) was 25 minutes.

Boehm noticed that in the gastrocnemius of *R. esc.*, ·005 p.c. nicotine causes a contraction which is not much weaker than that caused by ·1 p.c. In *Rana temp.* I find that with a load of 5 grams there may be no difference in the height of contraction with ·005 and ·1 p.c., but the rate of rise is slower with the lower percentage (Fig. 29). With a load of 8 grams, the rise is much slower with ·005 p.c. than ·1 and the height is less (Fig. 30). According to Boehm the rate of contraction only varies when very dilute solutions of nicotine are used. I find that whilst with a load of 5 grams the increment in the rate of rise decreases from the weakest effective nicotine solution up to ·1 p.c.; 1 p.c. nicotine distinctly increases the rate of rise (Fig. 29) and increases somewhat the height of contraction.

It will be noticed in the curves that the sooner the maximum height is attained, i.e. the greater the percentage of nicotine, the sooner the

fall begins. A similar phenomenon has already been shown in the flexor carpi radialis (Part II, p. 178) and the sartorius (cp. above Fig. 3).

When the muscle is immersed in successive solutions of nicotine of increasing strength, there is little or no distinction of primary and secondary rise as there is in the sartorius (cp. Figs. 4 and 5, Part III). The rise though somewhat quicker at first, is not (with a load up to 8 grams) followed by a fall before the rigor rise. It will be noticed in Fig. 28 that ·1 p.c. nicotine after ·005 p.c. causes no rise in the curve, this indicates that the ·005 p.c. solution had paralysed the receptive substance, and that ·1 p.c. did not stimulate the general muscle substance.

Boehm states that the gastrocnemius contracts with nicotine if curari is injected into the frog, though it does not contract if the muscle is cut out and soaked in curari. I find that if ·5 c.c. of 1 p.c. curari is injected into the dorsal sac of a frog with the brain destroyed, and it is left for an hour, nicotine ·005 p.c. has no effect, but ·1 p.c. causes a slow contraction (Fig. 31). After injection of 1 c.c. of 1 p.c. curari,

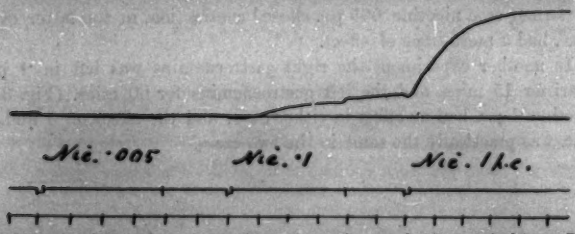

Fig. 31. × ½. Effect of nicotine on the gastrocnemius muscle of a curarised frog. Brain destroyed, ·5 c.c. 1 p.c. curari injected into dorsal sac, muscles taken after 1 hour. Load 1½ grams.

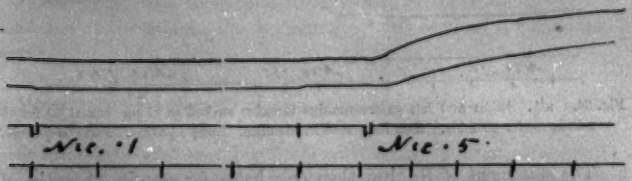

Fig. 32. Right and left gastrocnemius muscles, 2 hours after injection of 3 c.c. 1 p.c. curari. Load 1½ grams. Time in ½ mins. There is an interval of 3 mins. between the two parts of the tracing.

·1 p.c. nicotine gives a mere trace of contraction with a load of 8 grams but still gives one with a load of 1½ grams. After injection of 1 c.c. of 1 p.c. curari into the dorsal sac and of 2 c.c. into the abdominal cavity, the muscle being taken after 2 hours, ·1 p.c. nicotine has no effect, but a contraction is obtained with ·5 p.c. and with 1 p.c. (Fig 32).

Fig. 33. ×½. (a) Left gastrocnemius in curari ·001 p.c. ½ hr. (b) Right gastrocnemius in curari ·01 p.c. 1 hour. Load 1½ grams. Time in ½ mins. Between the two pieces is an interval of 3 mins.

On soaking the cut out muscle in curari, the same gradual abolition of the effect of nicotine is observed. In Fig. 33 the gastrocnemius of one side (*a*) was soaked in ·001 p.c. curari for ½ hour, and the gastrocnemius of the other side (*b*) in ·01 p.c. curari for an hour. In the former case nicotine ·005 p.c. caused contraction, in the latter even ·1 p.c. had a mere trace of effect.

In another experiment the right gastrocnemius was left in ·1 p.c. curari for 15 mins. and the left gastrocnemius for 60 mins. (Fig. 34). Nicotine ·1 p.c. had no effect in either case; and the effect of ·5 and of 1 p.c. was practically the same in the two cases.

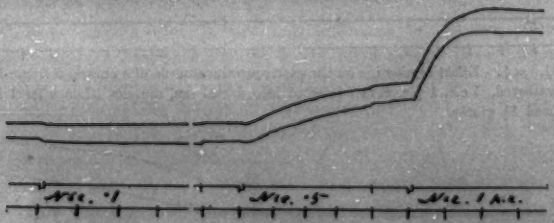

Fig. 34. ×½. Right and left gastrocnemius muscles soaked in ·1 p.c. curari (*a*) for 15 mins., (*b*) for 60 mins.; then immersed in nicotine. Load 1½ grms. Time in ½ mins.

The statement of Boehm then only holds for certain strengths of curari and nicotine. After injection of a sufficient dose of curari the gastrocnemius does not contract with nicotine up to ·1 p.c. but does

with ·5 and with 1 p.c.; and after immersion of the muscle in curari, a contraction is still obtained with ·5 and with 1 p.c. nicotine.

In the foregoing experiments, all the muscles gave the more prolonged form of curve spoken of above. It is clear that in these cases the gastrocnemius differs in type from the sartorius and more nearly resembles the coraco-radialis (cp. Part I, p. 376) and the flexor carpi radialis (cp. Part II). Its receptive substance requires a much higher percentage for complete paralysis than is the case with the sartorius. The difference in this respect in different muscles of the frog I have mentioned in my Preliminary Communication[1].

I have also pointed out that the greater the amount of curari required to paralyse the receptive substance in a muscle, the smaller the percentage of nicotine which will stimulate it. In accordance with this rule, the gastrocnemius gives a fairly good, though slow, contraction with ·0001 p.c. nicotine (Fig. 35). We have seen that in the sartorius the fibres do not all respond equally with nicotine. It is possible that

Fig. 35. (*a*) Left gastrocnemius taken out of body 7 minutes after death. (*b*) Right gastrocnemius taken out of body 1 hr. after death. Each immersed in nicotine ·0001 p.c. The steps at the end of the curve mark intervals of 15 mins. in the tracing. The time from immersing in nicotine to the first step was 5 mins. Load 1½ grams.

in the gastrocnemius, there are a varying number of fibres of the type of the sartorius; that their presence accounts for the early partial fall which, as I have said at the beginning of this section, is sometimes observed.

Boehm found in the gastrocnemius that curari sometimes, but not constantly, increased the rate of relaxation, and he states that it is not obtained if nicotine above ·05 p.c. is used. I take it that curari always causes relaxation after nicotine, if the contraction is being maintained at the time by stimulation of the receptive substance. I have obtained a distinct fall after ·1 p.c. nicotine (Fig. 36).

[1] This *Journal*, XXXVIII. 1909 (*Proc. Physiol. Soc.* p. lxxi).

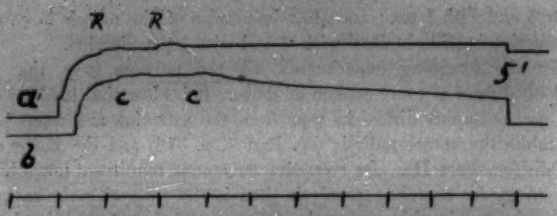

Fig. 36. Right and left gastrocnemius, (b) was taken 24 mins. after (a). The vessel containing the muscle was first filled with nicotine ·1 p.c. After 30 secs., this was run off, and Ringer's fluid poured in (a), and curari ·1 p.c. in (b). In 30 to 45 more secs. the fluid in each was poured off and renewed.

The absorption of nicotine and curari by muscle and other tissues.

I have said earlier that the sartorius gives a slight tonic contraction with about ·001 p.c. nicotine, the flexor carpi radialis with about ·0001 p.c. and the rectus abdominis with about ·00001 p.c.[1] The effect of the nicotine in each case is prevented by a definite amount of curari. The muscles then form a series which can be used to determine the amount of nicotine or curari contained in a tissue extract provided allowance is made for any stimulating or paralysing action of the other substances in the extract. A brief account of some of the experiments I have made on these lines may be given here, though I shall treat the matter more fully at a later time.

The tissue extract was made by placing the tissue in Ringer's fluid (generally in the proportion of 1 gram of tissue to 10 c.c. of fluid), boiling, grinding—mincing if necessary—boiling again and filtering. In testing this for nicotine a portion of fibrate is dropped over the rectus abdominis muscle arranged for giving a graphic record, the fluid is collected in a watch glass placed under the muscle, and sucked up with a pipette, dropped in the muscle again and so on for 5 mins. The extract of a fresh unpoisoned tissue 1 in 10 causes a trace of contraction only. In testing it for curari a sartorius muscle is immersed in it for 15 to 30 minutes, and then a graphic record taken of the effect on the muscle of ·1 p.c. nicotine.

Proceeding in this way I find that striated muscle, unstriated muscle, liver and kidney on being placed in a nicotine solution for 15 mins. and

[1] *Proc. Physiol. Soc.* p. lxxi. 1909 (This *Journal*, xxxviii.).

then in Ringer's fluid for 30 mins. all contain an easily detectible amount of nicotine. If the tissue is boiled before being placed in nicotine, very much less is taken up.

The amount taken up increases with the percentage of nicotine in the solution in which the tissue is placed, except perhaps with very dilute solutions.

The amount taken up from a ·001 to ·01 p.c. solution is approximately the same for all the tissues tried.

The slowness of the extraction with Ringer's fluid I take to mean that the nicotine is in a state of 'adsorption.'

Curari does not prevent muscles from taking up nicotine.

Since nicotine action, as we have seen, depends more on the percentage than on the total amount, the fact that it is adsorbed by tissues generally must very greatly reduce its action on the receptive substance, and so protect the specially sensitive tissues.

I have made fewer observations on curari but the general results have been the same, though various tissues not known to be specially acted on by curari apparently took up more curari than either the sartorius or the rectus abdominis.

Summary and Conclusions.

Unless otherwise mentioned the results were obtained on the sartorius muscle.

The minimal percentage of nicotine which causes twitchings and slow tonic contraction is about ·001 p.c. at 13°—17° C.; the percentage decreases as the temperature increases.

The height of the tonic contraction increases with increase of percentage from ·001 to ·1 p.c. nicotine; the difference in height is but slight in passing from ·1 to ·5 p.c., but is large in passing from ·5 to 1 p.c.

Other things being equal, the quicker the maximum height is attained, the sooner the fall sets in; with ·5 to 1 p.c. nicotine the fall soon passes into the slow rise of rigor, the curve of rigor when established is convex to the abscissa. The rise depends upon continuous immersion in nicotine, and a slow fall is obtained by transferring the muscle to Ringer's fluid.

Dilute nicotine decreases the effect of a stronger solution; after brief action of ·01 p.c. or longer action of ·001 p.c., ·1 p.c. nicotine has no

immediate effect and the immediate effect of 1 p.c. is much reduced. Nicotine ·0001 p.c. has a very slow paralysing action.

Nicotine ·01—·1 p.c. in time causes a slow rigor rise, the time depending greatly on the temperature. Sometimes, apparently, at 22°—24° C., ·1 p.c. nicotine causes a slight immediate rise by an action on general muscle substance, and this slowly passes into the rigor rise. Local contraction can be obtained by strong electrical stimuli in a late stage of the rigor rise.

The muscle fibres differ greatly in their vitality and they differ also in their reaction to nicotine; the fibres of the inner surface give a stronger tonic contraction than those of the outer surface, and the rigor contraction is greater in them.

The salts contained in a 1 p.c. solution of curari, acting on the muscle for 30 mins., do not prevent the effect of ·1 p.c. nicotine, but they have some action since they reduce the effect of ·01 p.c. nicotine. The salts contained in a ·25 p.c. solution of curari have in 30 mins. no appreciable effect. Curari ·25 to 1 p.c. gradually reduces in proportion to the percentage the electrical excitability of the muscle.

On immersing the muscle in curari for 15 mins. and then in nicotine it is found that a much less percentage of curari than ·0001 will prevent the effect of ·001 p.c. nicotine; as the percentage of curari is increased, a higher percentage of nicotine is required to cause contraction. It is on a similar relation of the amount of paralysing poison to the amount of stimulating poison that most physiological antagonisms obtained in the body rest. From a comparison of the effects of curari and nicotine on the different muscles of the frog, we may infer that the range of physiological antagonism between any stimulating and paralysing poison is greater the greater the responsiveness of the receptive substance to the stimulating poison.

After a certain percentage of curari, evidence of antagonistic action is less obvious. Thus after the sartorius has been soaked for 15 mins. in ·001 p.c. curari, ·1 p.c. nicotine has no immediate effect upon the receptive substance, nor does ·5 p.c. appear to have any. But so far as the experiments go, 1 p.c. has a slight immediate effect. This I take to be due to the substitution of nicotine for curari in the combination formed with the receptive substance. Below ·001 p.c. curari there is evidence that a slow substitution takes place.

The paralysis by curari of the twitchings and of the tonic contraction caused by nicotine does not necessarily occur at the same time.

The time taken to paralyse the receptive substance to ·1 p.c. nicotine rapidly increases with percentages below ·0001.

The curari paralysis diminishes on leaving the muscle in Ringer's fluid, but does not disappear in a day. The rate of disappearance decreases as the percentage of curari increases.

No evidence was obtained of an action of curari up to about ·1 p.c. in 15 mins. on the general muscle substance, but on longer stay in ·1 p.c. or in stronger solutions, the primary rise with ·5 to 1 p.c. nicotine increases, there is a slight primary effect with ·1 p.c., and the secondary rise is slower.

Curari given after nicotine stops the stimulating action of the latter, but has little if any effect upon the tonic contraction caused by the previous stimulus, *i.e.* it paralyses the receptive substance but has little if any effect on the general muscle substance whether this is at rest or contracted.

Curari ·001 p.c. was not found to have any certain effect on the slow loss of irritability caused by ·1 p.c. nicotine.

When mixtures of curari and nicotine are poured over a muscle, a slight contraction is obtained with ·1 p.c. nicotine in the presence of ·2 p.c. curari, and with ·01 p.c. nicotine in the presence of about ·02 p.c. curari.

On injecting curari into a frog, the effect of nicotine on the twitching ceases to be obtainable at about the time when contraction can no longer be obtained by nerve stimulation; but sometimes the former slightly outlasts the latter. The time at which tonic contraction ceases to be obtainable varies in different muscles; in the sartorius it is approximately the same as that at which paralysis of the nerve occurs, though usually it is a little later; in the flexor carpi radialis, the rectus abdominis and the gastrocnemius it is considerably later.

NaCl ·6 p.c. greatly increases the twitchings caused by nicotine, especially ·001 p.c.; no evidence was obtained that it increases the tonic contraction. Previous immersion for 30 mins. in ·1 p.c. nicotine does not prevent the occurrence of twitches in NaCl ·6, but it decreases them. KCl ·5 p.c. in Ringer's fluid soon prevents nicotine ·1 p.c. from causing contraction. A similar effect is produced by a strong solution of the salts of muscle. $CaCl_2$ ·5 p.c. in Ringer's fluid also prevents the action of ·1 p.c. nicotine, but more slowly.

$MgCl_2$ ·5 p.c. in Ringer's fluid does not in an hour prevent the effect of ·1 p.c. nicotine, though it considerably reduces the electrical irritability.

In the gastrocnemius muscle the general results are the same as in the sartorius, but the muscle responds to a much smaller percentage of nicotine, and its contraction is more prolonged. Since the duration of the plateau varies in different frogs, there is probably in the muscle a varying admixture of different kinds of muscle fibres.

Nicotine is adsorbed by all tissues; the amount taken up increases with the percentage in the fluid in which the muscle is placed. In the body this probably tends to decrease the poisonous action. Curari does not prevent the adsorption, and so far as the experiments go, curari is similarly adsorbed.

Nicotine ·25 to 1 p.c., after the receptive substance has been paralysed[1], frequently gives in the sartorius a curve in which there is a fall after the primary rise (cp. Fig. 23 *b*). Both the rise and the subsequent fall indicate an action on some special molecules in the general muscle substance, different from those which are acted on in producing the secondary (rigor) rise. I have previously suggested that the receptive radicle is present but in more stable chemical combination throughout the general muscle substance. The fact mentioned above favours this suggestion, on the other hand I have not found that curari within wide limits has any influence on the substance which gives the primary rise with 1 p.c. nicotine.

The experiments I think tend to support the theory that nicotine and curari form dissociable compounds with the receptive substance. The chief results may be stated in the terms of this theory:

The height of the contraction caused by the combination of nicotine with the whole of the receptive substance depends upon the rate of combination and on the duration of the resulting contraction; this dependence is chiefly, if not altogether, due to the degree of summation of contraction in all the muscle molecules. The saturation point is quickly reached in the sartorius with ·01 p.c. nicotine and then ·1 p.c nicotine has no effect. If combination is slow enough, and the duration of contraction in each muscle molecule brief enough, complete saturation of the receptive substance may occur without any visible contraction.

When the receptive substance is saturated with curari, and in some cases before complete saturation, nerve impulses cannot affect it.

The substitution of nicotine for curari in a curarised muscle is delayed by adsorbed curari.

[1] After the receptive substance has been paralysed by curari, 1 p.c. may cause some contraction by turning out the curari and stimulating the receptive substance. There would naturally be some fall after this contraction.

There appears to be a lower limit to the concentration of nicotine and of curari, which can saturate the receptive substance; this implies that the latter is combined with something of very weak affinity for it, but of just sufficient affinity to prevent poisons below a certain concentration from combining with it. The most probable combination of the receptive substance is with an ion, or a molecule, of neutral salt. On this view the action of poisons depends not only upon their relative affinities for the receptive substance, and on their relative concentration, but also on the relative affinity and concentration of certain ions or salt molecules.

CRITICAL REMARKS.

In the preceding account I have taken it for granted that nicotine and curari act on the same substance because it seems to me that no other explanation can be given of the facts. It is true that Magnus[1] a short time ago argued that the phenomena of mutual antagonism gives no basis for drawing any conclusions as to the point of action of poisons. But Magnus deals only with the theory that the specific action of poisons is one on the nerve endings, he does not consider my theory of the presence of more than one receptive substance in the cell. I need then only point out that if his argument is just, it affords a reason in addition for rejecting the theory of the specific nature of nerve endings.

Experiments on the fowl have been made by Edmunds and Roth. When their experiments were the same as mine they obtained the same results except that in long denervated muscles they obtained, in every case but one, less relaxation of nicotine contraction by injecting curari. They tried much more frequently than I did the effect of curari after nicotine and they found that from about the third day onwards curari had (with the one exception mentioned above) a mere trace of effect on the nicotine contraction. Their conclusion from this is that curari does not antagonise the action of nicotine in the denervated muscle. This conclusion is different from mine, which was that curari antagonised nicotine in the denervated as well as in the normal muscle, though a larger amount of curari was required. Edmunds and Roth quote two of my experiments as being in favour of their view. They have however overlooked what these experiments were designed to test and what they in fact show. I had pointed out that there were three ways of testing the antagonism of curari for nicotine.

[1] *Pflüger's Arch.* CXIII. p. 99. 1906.

(1) By giving curari primarily and seeing whether a normally effective dose of nicotine had its normal effect.

(2) By interpolating an injection of curari between two doses of nicotine, the second being given at a sufficient interval after the first to ensure that without curari it would produce an effect nearly if not quite as large as the first.

(3) By giving curari after nicotine.

These experiments of mine which are referred to by Edmunds and Roth were made on the second plan, not as supposed by them on the third. The experiments clearly showed that the injecting of curari greatly reduced the effect of the second dose of nicotine, *i.e.* that curari antagonised the action of nicotine. This result, combined with the slight effect obtained by plan 3, showed, though I did not appreciate it till later, that curari whilst preventing the stimulating action of nicotine does not, or only to a slight extent, undo the effects of the stimulus when this has been delivered.

The difference in the behaviour of the normal and of the denervated muscle as regards the amount of curari required to antagonise the nicotine action, I attributed to an increased irritability of the muscle to nicotine. This view Edmunds and Roth support, and give further evidence that the irritability is in fact increased. But the decreased effect of curari *after* nicotine in the denervated muscle is due in part to another factor, viz. a decrease in what may be called the relaxing power of the general muscle substance. An increase in contracture is a known feature of atrophying muscle.

Edmunds and Roth find in the denervated muscle a similar antagonism between curari and physostigmine on the muscles of the fowl to that which exists between curari and nicotine, adding thus another instance to the list of those which show that the action of poisons supposed to act specially on nerve endings is fundamentally the same whether nerve endings are normal or degenerated. The parallelism which exists in the behaviour of the muscles of fowl, and the muscles of the frog which respond to nicotine, shows I think definitely that in the former as in the latter the action of curari, nicotine and physostigmine is not on the general muscle substance but on the receptive substance.

Whilst speaking of Edmund and Roth's experiments I may note that they refer to Heidenhain[1] as if he had advocated the view that nicotine and curari do not act in motor nerve endings in the tongue

[1] Heidenhain. *Arch. f. (Anat. u.) Physiol.* 1888. Supp. p. 183.

muscles but on the 'sole' of the nerve endings. Heidenhain gave this view, as one of two alternatives[1], to account for the persistence of nicotine and curari action on the tongue muscles, after section of the hypoglossal nerve. The explanation of the phenomena observed after section of the hypoglossal nerve was at that time and so a certain extent still is obscure. But on the theory I have given, the phenomena are readily accounted for. I take the spontaneous twitchings which occur shortly after section of the hypoglossal as due to chemical stimulation of the receptive substance increased in excitability by nerve section, and the continuance of the nicotine and curari action as another instance that these substances act on the receptive substance and not on nerve endings.

Latterly some observers have suggested that variation in the permeability of the cell, or of the solvent power of its limiting layer (on the lines of Overton's and H. Meyer's results), may account for the specific action of poisons. The suggestions are not sufficiently formulated to make it worth while to consider them. But one definite suggestion has been made by Straub[1] in relation to the action of muscarine and atropine on the heart, and this it is necessary to discuss.

Straub finds in Aplysia that muscarine is stored up in the heart muscle; that a certain percentage in the outside fluid is required to produce inhibitory change; that if the muscarine in the surrounding fluid be removed, the heart at once begins to beat, and that after a certain amount has been absorbed the heart beats spontaneously and is unaffected by muscarine whatever quantity the fluid surrounding it contains. He suggests that inhibition is caused by the physical process of the passage of muscarine through the limiting layer of the cell and that when it has passed this layer it cannot cause inhibition. But Straub himself finds that this does not necessarily hold in the case of the frog's heart, inasmuch as inhibition may continue when he thinks absorption must have ceased. It is most unlikely that the laws of inhibition are different in these two cases, so that if Straub's conclusion for the frog is correct, a theory which is based on the assumption that inhibition is caused by absorption must be inadequate. On the chemical theory the muscarine must pass into the cell before it can combine, so that in general the inhibition will be produced during absorption, but on the chemical theory the inhibition is not necessarily restricted to the time

[1] Heidenhain says "Ohne für jetzt zwischen beiden Möglichkeiten, die eine kritische Erwägung der Thatsachen im Auge zu behalten hat, entscheiden zu wollen...." *Op. cit.* p. 166.

during which absorption takes plate, i.e. it is more in accordance with the facts so far as they are known. Straub as mentioned showed that in aplysia, the heart after absorbing a certain amount of muscarine was no longer affected although the percentage of muscarine in contact with the heart was greatly increased. If as he supposes, the absorption is due to a concentration difference inside and outside the cell, there seems no reason why a stage should be reached, apart from damage to the cell, at which this difference should cease to act. The aplysian heart beats normally at the stage at which concentration difference is supposed to have no effect. On the chemical theory the result can be readily explained, the receptive substance sets up a stimulus by combination; when it is saturated further muscarine has of course no effect.

Straub does not explain the mechanism by which absorption causes inhibition, nor why strychnine, which he finds is absorbed, does not cause inhibition. There are also other difficulties when the hypothesis is extended to meet other cases, such as the frequent paralysis of nerve effect by stimulating poisons, and the fact that the same substance, for example adrenalin, sometimes causes contraction and sometimes inhibition within the same class of tissue cells.

Straub has also dealt with the question of the mutual antagonism of poisons. He found in the Selachian heart that atropine though it did not prevent muscarine from being absorbed, delayed the rate of absorption. He suggests that atropine alters the limiting membrane, so that it is a worse solvent for muscarine and thus reduces the rate of muscarine absorption below the threshold velocity. An objection may be made to this view on the ground of the experiment itself, the reduction which atropine caused in the amount of muscarine absorbed was not great, and it seems to me insufficient to account for the total absence of inhibitory action. However this may be, the result is much more naturally accounted for by the hypothesis that atropine combines with the receptive substance and in consequence prevents muscarine from having any effect.

In the case of nicotine and curari Straub's hypothesis is I think inconsistent with the phenomena. In the sartorius muscle of the frog nicotine ·005 to ·1 p.c. causes a slow contraction when applied to the neural region of the muscle fibre but not when applied elsewhere. This, as we have seen, is an effect on the cell and not on the nerve ending. Nicotine ·25 to ·5 p.c. causes contraction in other parts of the muscle, but the contraction is of much less intensity than that caused by more dilute nicotine in the neural region and so can be distinguished from it. Curari ·001 p.c. prevents a contraction being obtained by nicotine ·1 p.c. but

curari ·25 p.c. does not appreciably affect the contraction caused by nicotine ·5 p.c. in the non-neural region. If Straub's hypothesis is applied to this case it follows that ·001 p.c. curari so alters the limiting layer of the muscle fibre that nicotine ·1 p.c. is practically unabsorbed, and yet that it alters it so little that ·5 p.c. nicotine is absorbed at a rate indistinguishable from normal. It might perhaps be said that the limiting layer is different in the neural and non-neural region, but this would be no way out of the difficulty. For after curari ·001, nicotine ·25 to ·5 p.c. produces at least as great a contraction in the neural region as it produces elsewhere, and this contraction is not diminished by increasing the curari up to ·25 p.c.

Another hypothesis has recently been put forward by Dixon and Hamill as far as regards the action of vegetable drugs. They suggest that vegetable drugs set free a hormone which combines with a receptive substance in the cell. In two essential points, viz. the existence of a receptive substance and the production of activity by a chemical combination with the receptive substance, this view is the same as mine. It differs in that the poison instead of itself combining with the receptive substance sets free from something else another body—an organic body comparable to secretin and adrenalin—which combines with the receptive substance. They consider that the same hormone is set free by nerve stimuli.

We may consider first the reasons they allege against the theory that vegetable drugs combine directly. The reasons are two (1) that strychnine when ground up with spinal cord can be extracted from the emulsion. This is directed against an argument used by Ehrlich in the case of toxin receptors, but it obviously has no force against the view I have given above, any more than the fact that oxygen can be extracted from oxy-hæmoglobin is evidence that oxygen does not combine with hæmoglobin. (2) The second is that a spinal cord emulsion has no specific effect in diminishing the physiological action of strychnine. This is based on their observation that a spinal cord emulsion does not diminish the lethal dose of strychnine more than it is diminished by other colloids. The explanation I think is that all colloids adsorb poisons, and that in the emulsion the adsorbed part is much larger than the chemically combined, so that the reduction of lethal action is approximately equal with a variety of colloids. A delicate test is then required to detect the difference due to chemical combination, and the injection of a given quantity per kilogram of body weight into different animals can hardly be called a delicate method.

Further the argument assumes that the receptive substance can combine after death. It is possible that the receptive substance becomes altered on the death of the cell so that it no longer combines with poisons.

The hypothesis of Dixon and Hamill is more complicated than that which I have given, since it requires the presence of an additional substance in the cell capable of liberating a hormone. And it is less complete since it does not explain how the hormone is liberated. They say that drugs 'like enzymes and catalytic agents take no part of a chemical nature in the ultimate changes,' but they do not say that drugs are catalytic agents. A theory based on catalysis (though it would be simpler without the supposition of hormones) might find some support from the adsorption phenomena I have described; but it has grave difficulties, chief amongst them perhaps that of attributing an anti-catalytic agency to curari and other similar poisons.

On Dixon and Hamill's hypothesis adrenalin and the active substance of other internal secretions correspond to the primary products caused by nerve impulses, and not, as generally held, to the final products. It may fairly be argued that if in glands and muscle, the primary products are produced by nerve impulses, there would be a specially developed nerve supply in those tissues in which the formation of such products are specially developed. In fact there is no clear evidence that the glands of internal secretion are influenced by nerve impulses at all.

Dixon and Hamill's view of the method of action of secretin also differs from mine. They find that secretin combines chemically with a pro-zymogen in the pancreas and converts it either into zymogen or into enzyme, and they consider that in doing this it sets up secretion. It seems to me unlikely that secretion is caused by this process, and more probable that it is due to the combination of secretin with a receptive substance (an atom-group) of the living protoplasm.

In this paper I have not discussed my hypothesis that the receptive substance is an atom-group of the protoplasm, but I may say a word or two about it.

I assume that all protoplasm has a number of atom-groups which can combine with this or that chemical substance. The degree to which these are essential parts of the molecule varies, but two groups may be distinguished.

In one group the linkage of the atom-groups is such that they can be combined so as to be thrown out of gear (and probably split off), without injury to the rest of the molecule. Their chemical combination may however lead to those changes in the whole molecule normally associated with its function. These are the receptive atom-groups. In less differentiated cells the existence of such atom-groups is indicated by the formation of anti-bodies. In more differentiated cells, a special

development of receptive atom-groups takes place and it is in these that secretin, adrenalin, curari, nicotine etc. act. When a nerve fibre ends in connection with the cell, the receptive atom-groups are in some cases, and perhaps in all, more or less localised in the region of the nerve endings.

In the other group, the linkage of the atom-groups is such that they cannot be split off or combined without causing serious alteration in the whole molecule. These are essential to the proper working of the mechanism, and they may be called the fundamental atom-groups.

Whilst we can refer a large number of chemical actions to one or other of these two groups, there are some, for example the action of salts and ions on the general muscle substance, which cannot at present be definitely referred to either, and some, for example that of digitalis on the heart, in which the combination seems to aid the mechanism.

This hypothesis is an extension of Ehrlich's side chain hypothesis, though it has been reached by entirely different experiments and by a different line of argument. In 1906 I suggested that the action of drugs might be referred to a combination with side chain radicles. At that time Ehrlich had not brought these bodies into his scheme. Since then he has done so. The evidence he gives is based on the action of various arsenic compounds on the trypanosomes of sleeping sickness. The particular protoplasmic groups which combine with chemical bodies of the drug type he calls chemo-receptors. It will be noticed from what I have said above that the chemo-receptors of Ehrlich include atom-groups which do not belong to my receptive class. For the combination of arsenic compounds with the chemo-receptors of the trypanosomes leads to the destruction of the organism, *i.e.* the atom-groups acted on are fundamental and not receptive.

ON THE ESTIMATION OF PURINE BASES IN URINE.

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In the course of some work on the subject of endogenous uric acid[1], it was found that the figures obtained by the Camerer-Arnstein method for the total purine nitrogen of urine were in many cases lower than those representing the nitrogen of the uric acid alone; in the remainder there was great irregularity, the total purine nitrogen often exceeding the uric acid nitrogen by but a very small amount. In the series of observations published by Macleod and Haskins[2], who employed the same methods of estimation, the total purine nitrogen is on some occasions but slightly greater than the uric acid nitrogen, and on two occasions is lower than it.

At Dr Leathes's suggestion, some experiments were carried out to test the accuracy of the Camerer-Arnstein method.

The Camerer-Arnstein method.

The analysis is carried out in the following way[3].

The phosphates of the urine are removed by means of magnesia mixture and ammonia. Ammoniacal silver nitrate is added to the filtrate; the precipitate is filtered off by means of the pump, the last portions being washed on to the filter with weak ammoniacal water. The precipitate is then washed with water until the filtrate is neutral to litmus paper. The precipitate and filter paper are then transferred to a Kjeldahl flask, and boiled with water and magnesium oxide till very little water is left (Neubauer and Vogel, *Analyse des Harns*, 10th ed. p. 817). The amount of nitrogen present is estimated by the Kjeldahl process. In the method as described originally[3] the precipitate was not boiled before the Kjeldahl estimation. Salkowski[4] pointed out that these precipitates contain ammonia even after prolonged washing. Subsequently Arnstein recommended boiling with magnesium oxide as a means of removing this ammonia, and stated that he obtained the correct amounts of nitrogen when testing the method, as thus modified, with known weights of uric acid[5]. He does not bring forward these results.

(*a*) *Results obtained with uric acid.*

As uric acid is the purine present in largest amount in urine, and as it seemed to be the most likely to undergo decomposition when its silver compound was boiled, it was used in the first experiments.

(1) Weighed amounts of uric acid were dissolved with caustic soda. Acetic acid was added until the uric acid began to be precipitated. Ammonia was then added until complete solution was obtained; by this means, the presence of an excess of caustic soda was avoided. The uric acid was precipitated with magnesia mixture and ammoniacal silver nitrate; in order to secure as complete precipitation as possible the amounts of silver and magnesium added were about three times as great as those calculated to be necessary[1]. The mixture was in all cases allowed to stand overnight before filtering. To remove the ammonia, the precipitates were washed till the filtrates were neutral and free from chlorides and nitrates, and were then dried for five hours at 65° C. The pump was not used in filtering.

	Calculated	Found	Loss per cent.	
Unboiled ...	22·90 mg. N	20·93	8·6	Mean 4·9
	26·66	25·92	2·8	
	22·27	21·56	3·2	
Boiled 2 hours ...	25·93	22·26	14·2	
	31·90	25·67	19·5	
Boiled 2 hours with MgO	13·57	11·48	15·2	Mean 13·2
	24·43	22·47	8·1	
	35·40	32·13	9·0	
Boiled nearly to dryness with MgO	21·70	18·76	13·5	
	24·53	21·65	11·7	

(2) Two precipitates were prepared as described above, but were not dried before boiling. Arnstein's directions as regards amount of nitrogen, time of standing before filtration, method of filtration, and time of boiling, were followed.

Calculated	Found	Loss per cent.
15·07 mg. N	12·95	14
11·33	9·73	14

(3) A number of experiments were carried out in a similar manner with silver urate, no magnesia mixture being used; about the same average loss (13 %) occurred in the boiled preparations.

No relation could be observed between the duration of boiling and the amount of loss.

Duration of boiling	Calculated	Found	Loss per cent.
15′	29·40 mg. N	24·94	15·2
30′	33·33	29·61	11·2
60′	27·58	23·74	13·8

(4) A number of estimations were carried out in which silver urate or silver magnesium urate was boiled with magnesium oxide, the mixture then treated with sodium or hydrogen

[1] When precipitated by these solutions, uric acid is combined in the proportion of one molecule to one atom of each of the two metals (Neubauer and Vogel, *op. cit.* p. 318).

sulphide, and the uric acid present estimated gravimetrically as in the Salkowski-Ludwig process. The boiled preparations, in comparison with the controls, showed a loss of about 40 % of the weighed amount of uric acid present at the outset. This method could not, of course, demonstrate that any loss of nitrogen occurs in boiling.

These experiments indicated that the low results obtained by the Camerer-Arnstein method were due chiefly to decomposition of uric acid during boiling. Accordingly, in further experiments, the influence of boiling was eliminated.

(5) The precipitate when washed neutral was placed in a combustion flask with magnesium oxide and kept at 37° C. and 30 mm. Hg pressure till apparently dry.

In one case, this dry residue was extracted with water, and the amount of ammonia present estimated by Nessler's reagent; 0·05 mg. ammonia was found.

	Calculated	Found	Loss per cent.	
(*a*)	26·10 mg. N	25·27	3·2	
(*b*)	22·67	20·72	8·6	
(*c*)	25·03	23·77	5·0	Mean 4·9
(*d*) *In vacuo* when all on filter. Not washed neutral ...	20·73	20·16	2·8	

The average loss in these cases is the same as in those described above (p. 297) in which the precipitates were not boiled. Since the method here employed to remove ammonia was shown to be effectual, the higher result of estimation (*d*) is probably due to the absence of prolonged washing with consequent solution of some of the precipitate.

It was thought possible that there might be some evolution of free nitrogen during boiling, owing to the action upon the uric acid of nitrite formed from the silver nitrate by reduction. Magnesium silver urate was therefore boiled with magnesium oxide in a stream of CO_2 which passed into a nitrometer filled with potash. No nitrogen was obtained. Moreover, subsequent experiments showed that the ammonia-nitrogen given off during boiling gave with the nitrogen found in the precipitate and filtrate a total which was approximately equal to the amount of nitrogen present at the outset.

If the filtrates from the precipitate of magnesium silver urate be kept for a few days, a further quantity of the flocculent white precipitate generally appears in them. An attempt was made therefore to estimate the fractions of the total loss due respectively to incomplete precipitation and to decomposition during boiling. With this object the ammonia contained in the precipitate was by various means removed as completely as possible; the precipitate was then boiled, and the distillate received in $\frac{N}{10}$ H_2SO_4. In some cases the filtrate was kept for two or more days, the precipitate in it then filtered off, and together with the filter paper

boiled with magnesium oxide to remove ammonia; the nitrogen present was then estimated by the Kjeldahl process.

(6) Uric acid dissolved with sodium carbonate.

Three portions of the solution precipitated with magnesium chloride and ammoniacal silver nitrate, precipitates washed neutral to litmus, placed in a vacuum desiccator over H_2SO_4 at 37° for 10 days, re-exhausted twice. At the second exhaustion, a piece of moist litmus paper was placed in the desiccator, and an evolution of ammonia observed by this means; at the third exhaustion, this test gave a negative result. Precipitates boiled with MgO for 1½ hours.

Precipitates	Distillate	
89·60 mg. N	4·40	(1st 30′)
	0·75	(2nd 30′)
	0·65	(3rd 30′)
	5·80	mg. N = 6·1 % of 89·60 + 5·80.

A portion of another solution was treated in the same way as described above, and boiled with MgO for two hours.

Precipitate	Distillate	
28·28 mg. N	2·28	(1st hour)
	0·46	(2nd hour)
	2·74 mg. N = 8·8 % of 28·28 + 2·74.	

(7) Uric acid dissolved with sodium carbonate.

Solution, Kjeldahl { 33·01 mg. N in 100 c.c.
33·15

Precipitated with magnesium chloride and ammoniacal silver nitrate. Precipitate washed neutral to litmus, and suspended in water in a combustion flask; the flask was kept at 35° C., and a current of air passed through the liquid. The ammonia which came over was received in $\frac{N}{10}$ H_2SO_4, which was titrated at intervals. After 6 hours, no more ammonia was obtained. The precipitate was then boiled with magnesium oxide for two hours.

Precipitate		Filtrate	Distillate	
28·70	+	1·54	1·62	(1st hour)
			0·60	(2nd hour)
			+2·22 = 32·46	

(2·22 = 7·2 % of 28·70 + 2·22).

Ammonia is therefore formed during boiling. This can be shown more satisfactorily if no ammoniacal reagents be used in the preparation of the precipitate. In the following experiments, magnesium sulphate and silver nitrate were used for precipitation.

(8) 1·25 gr. uric acid was mixed with enough 2 % caustic soda to convert 1 gr. uric acid to acid sodium urate. The mixture was left overnight, filtered, and made up to 500 c.c. The results showed that 0·849 gr. uric acid had dissolved. By this means, the presence of

a large excess of soda was avoided; the washings from the precipitate were neutral. Any caustic soda remaining adherent to the precipitate would have caused destruction of uric acid during boiling. Sodium carbonate cannot be used if silver nitrate is to be added.

Solution of acid sodium urate.

A. 50 c.c. Kjeldahl $\begin{cases} 27{\cdot}58 \text{ mg. N} \\ 27{\cdot}58 \end{cases}$

B. 50 c.c. precipitated with $MgSO_4$ and $AgNO_3$.

		Precipitate		Filtrate	Distillate		
(*a*)	... ...	26·95	+	0·63			= 27·58
(*b*)	Boiled 2 hours with MgO	25·27	+	0·35	0·91	(1st hour)	
					0·91	(2nd hour)	
					+1·82		= 27·44
		(1·82 = 6·7 % of 25·27 + 1·82).					
(*c*)	Boiled 1½ hours with MgO	25·69	+	0·63	0·84	(1st 30′)	
					0·70	(2nd 30′)	
					0·49	(3rd 30′)	
					+2·03		= 28·35
		(2·03 = 7·3 % of 25·69 + 2·03).					

Estimation (*a*) above indicates that if prolonged washing, and boiling, be avoided, the only source of error in this method is incomplete precipitation.

It was thought possible that the presence of small amounts of ammonia, such as are present when the method is used in the ordinary way, might facilitate the decomposition of the silver compound. Accordingly, in estimations (*b*) and (*c*) above, known amounts of a very weak titrated solution of ammonia were added before the final period of boiling, and these amounts subtracted from those found in the distillate. The percentage loss is, however, about the same as on other occasions.

The results given below afford further evidence of the increased accuracy of the method when boiling is avoided. A blank nitrogen estimation made upon the amounts of magnesium sulphate and silver nitrate present in these filtrates yielded the same figure that was used throughout this investigation (0·3 c.c. $\frac{N}{10}$ NH_3).

(9) Uric acid dissolved with caustic soda as acid sodium urate, as in (8) above. Precipitation with $MgSO_4$ and $AgNO_3$.

Solution 1. Kjeldahl. $\begin{cases} 31{\cdot}36 \text{ mg. N in 100 c.c.} \\ 31{\cdot}36 \end{cases}$

Precipitate		Filtrate		
31·22	+	0·35	=	31·57
31·22	+	0·70	=	31·92
31·15	+	0·49	=	31·64

Solution 2. Kjeldahl. $\left.\begin{matrix}30{\cdot}31\\30{\cdot}31\end{matrix}\right\}$

Precipitate		Filtrate		
29·12	+	0·91	=	30·03
29·05	+	0·91	=	29·96
29·19	+	0·98	=	30·17

The experiments described above showed that when magnesium silver urate is boiled a loss of nitrogen occurs which amounts to from 6 to 9 %. This loss does not occur unless the uric acid be combined with the metals. Berthelot and André[8] found that uric acid gave off no ammonia when boiled for an hour with magnesium oxide. In the present series, an experiment was made in which 0·2286 gr. uric acid was boiled for 75 minutes with silver hydrate and magnesium oxide; the amount of ammonia obtained was equivalent to only 0·8 % of the nitrogen of the uric acid.

(*b*) *Results obtained with purine bases.*

It was now necessary to ascertain whether the silver compounds of purine bases[1] undergo this loss during boiling.

(1) *Hypoxanthin, ammoniacal solution.* Two portions precipitated with ammoniacal silver nitrate; precipitates washed till neutral and free from chlorides and nitrates.

(*a*) Kjeldahl, 17·92 mg. N.
(*b*) Boiled two hours with MgO, 17·64.

(2) *Hypoxanthin.* (Contained 40·69 % nitrogen, calculated 41·18 %.) Two weighed amounts dissolved with ammonia, precipitated with ammoniacal silver nitrate, washed till washings gave very slight Nessler reaction.

	Calculated	Found
(*a*) Kjeldahl	39·75 mg. N	39·55
(*b*) Boiled two hours with MgO	64·61	63·98

(3) *Purine bases of urine.* The phosphates and uric acid of urine were precipitated with magnesia mixture, ammonia, and ammonium sulphate. The filtrate was precipitated with ammoniacal silver nitrate. The precipitate was washed on a number of small filters until 50 c.c. of the mixed filtrates contained 0·07 mg. ammonia, and was then decomposed with H_2S. Portions of the solution were precipitated with silver nitrate, no ammonia being used. In some cases the precipitation and filtration were carried out in a dark room. The filtrates from each pair of precipitates were mixed, and the nitrogen present estimated. The distillate from the precipitates which were boiled was received in $\frac{N}{10}$ H_2SO_4.

[1] Purine bases when precipitated by ammoniacal silver nitrate combine with a group Ag_2O (Neubauer and Vogel, *Analyse des Harns*, 10th ed. p. 340). No statement is made here as to combination with magnesium.

(A) Solution. Kjeldahl. 20·14 / 19·79 } 19·97 mg. N

Silver nitrate precipitates:

	Precipitate		Filtrate	
(B)	18·16 / 18·44 }	+	1·54	= 19·70 / = 19·98
(C) Boiled 25′ with MgO	18·23 / 19·01 }	+	1·73	= 19·96 / = 20·74
(D) ... ... One lost.	19·36	+	0·49	= 19·85
(E) Boiled 25′ with MgO	18·87 / 18·87 }	+	0·78	= 19·65 / = 19·65

(D), (E). Kept in dark room.

(C), (E). No ammonia evolved during boiling.

The silver compounds of the purine bases which were examined did not therefore undergo the decomposition during boiling which occurs in the case of uric acid.

A MODIFICATION OF THE CAMERER-ARNSTEIN METHOD.

Since uric acid was found to undergo loss when treated by the Camerer-Arnstein method, while the purine bases which were examined were not found to undergo this loss, the Camerer-Arnstein procedure was employed for the estimation of purine bases in urine after the removal of the uric acid.

Method A.

This method has been used in three series of observations (Cathcart, Kennaway, and Leathes, *Quart. Journ. Med.* I. p. 416, Protocol X.; Kennaway, *Journ. of Physiol.* XXXVIII. 1).

The estimation of uric acid is carried out by the method of Folin and Schaffer[7]; the ammonia added must be measured accurately, and the mixture kept during the precipitation in a closed vessel to prevent evaporation.

If 100 c.c. of urine be taken for the estimation, 100 c.c. of the filtrate obtained after the addition of the ammonium sulphate solution is placed in a 200 c.c. Erlenmeyer flask. 10 c.c. of 15 % ammonia is added. When the ammonium urate is filtered off, 100 c.c. of the filtrate is taken for the estimation of purine bases. To this are added 10 c.c. of 20 % ammonia and 10 c.c. of magnesia mixture. The phosphates are filtered off, and 10 c.c. ammoniacal silver nitrate added to 100 c.c. of the filtrate; the mixture is allowed to stand overnight, and the estimation completed as in the Camerer-Arnstein method.

The 100 c.c. of filtrate to which the silver nitrate is added represents $\frac{1}{2}$ of $\frac{11}{12}$ of $\frac{11}{12}$ of 100 c.c. of urine, that is, 60·6 c.c. of urine. It contains $\frac{11}{12}$ of the 3 mg. of uric acid stated by Folin and Schaffer to escape precipitation. From the amount of nitrogen found,

the nitrogen of this uric acid, namely 0·83 mg., must be subtracted; the resulting figure, multiplied by $\frac{100}{60-6}$, or 1·65, gives the amount of purine base nitrogen present in 100 c.c. of urine.

The precipitation of any of the purine bases together with the uric acid would of course introduce an error into this method. Hopkins[8] found that xanthin was precipitated by saturation of an ammoniacal solution with ammonium chloride. With a mixture of uric acid and a small proportion of xanthin, the latter was precipitated to a large extent by saturation with ammonium chloride, but the whole of it was found in solution when the uric acid was liberated. Hypoxanthin was not precipitated in this way.

In Beilstein's *Hdb. d. organ. Chemie* it is stated (B. III. S. 962) that xanthin dissolved by means of potash is not precipitated by ammonium chloride.

Edmunds[11] found that saturation of solutions of hypoxanthin and xanthin with ammonium sulphate produced no precipitate.

Folin[9] could not observe any precipitation of xanthin by ammonium chloride. He found guanin to be precipitated by ammonium chloride, carbonate, and sulphate, but not by the acetate[1].

This source of error in method A was examined as follows.

A solution of purine bases was prepared from urine. The phosphates of the urine were removed with ammonia and barium nitrate. The purines were precipitated with ammoniacal silver nitrate; the precipitate was washed and decomposed with hydrogen sulphide. The filtrate was evaporated to a small volume after addition of hydrochloric acid, and the uric acid filtered off. The filtrate was precipitated with silver nitrate, and the processes of decomposition and evaporation carried out as before; after this second evaporation, no separation of uric acid could be observed.

The amount of purine base nitrogen in the solution was ascertained by precipitation with ammoniacal silver nitrate. Other portions of the solution were treated as follows: (*a*) Folin and Schaffer's ammonium sulphate solution, and ammonia, were added in the proportions used in uric acid estimations, (*b*) ammonium sulphate solution and ammonia were added in amounts producing a concentration of 10 % of ammonium sulphate in the whole mixture. In both cases, the mixtures stood overnight and were then filtered. The purine bases in measured fractions of the filtrates were precipitated with ammoniacal silver nitrate; the filter papers were boiled with magnesium oxide, and the nitrogen present estimated. The following results were obtained.

[1] This precipitation of purine bases by ammonium salts might introduce an error into Wörner's method for the estimation of uric acid (*Zeit. f. physiol. Chem.* XXIX. p. 70. 1900). Wörner concludes that no such precipitation occurs, but he offers no evidence in support of this conclusion.

	Purine base solution		Precipitate		Total
(1) Original solution	6·80 mg. N 6·79 6·72	Mean 6·79			
(2) After treatment with Folin and Schaffer's solution (one lost) ...	6·12	+	0·28	=	6·40
(3) After treatment with ammonium sulphate 10 %.	6·27	+	0·42	=	6·69
	6·13	+	0·63	=	6·76

These results indicated that about 10 % of the purine bases was precipitated by the solutions used. It was thought that this result might be due to the presence of some uric acid in the purine base solution; accordingly, a similar experiment was carried out upon a portion of the solution which had been treated with manganese dioxide in the manner employed by Krüger and Schmid for the oxidation of uric acid (Hoppe-Seyler and Thierfelder's *Hdb.* 7th ed. p. 436).

	Purine base solution after oxidation		Precipitate		Total
(1) Original solution	4·41 mg. N				
(2) After treatment with Folin and Schaffer's solution.	3·82	+	0·59	=	4·41
	3·79	+	0·56	=	4·35

The loss here was even greater than it had been before oxidation.

It appeared then that the figures obtained by method A for the purine bases must be too low; the method described below was designed to avoid the source of error.

Method B.

In Hopkins's method of uric acid estimation, any purine bases precipitated by the ammonium salt should be in solution in the acid mother-liquor from which the uric acid separates. Attempts were made therefore to employ this method in the estimation of purine bases.

The results obtained by precipitating known weights of uric acid with ammonium chloride were not satisfactory. Moreover, it was observed later that ammoniacal silver nitrate does not precipitate the purine bases in urine saturated or half-saturated with ammonium chloride[1].

In the following experiments, the 50 % ammonium sulphate solution of Folin and Schaffer[7] was used.

(1) Uric acid dissolved with sodium carbonate, made up to 500 c.c.

(a) 150 c.c. Kjeldahl. 68·31
68·31 } mg. uric acid in 150 c.c. = 22·77 mg. in 50 c.c.

[1] This result suggests that ammoniacal silver nitrate cannot be used to demonstrate the complete precipitation of uric acid by ammonium chloride.

(b) 50 c.c. + 50 c.c. water + 25 c.c. Folin-Schaffer solution + 5 c.c. ammonia, stood overnight. Precipitates washed twice with 10 % ammonium sulphate and titrated.

22·50 mg.
22·71
22·87
23·25
} Mean 22·84 mg. in 50 c.c.

(2) Weighed amounts of uric acid dissolved with sodium carbonate in 100 c.c. water. 25 c.c. Folin-Schaffer solution and 5 c.c. ammonia added. Stood overnight. Precipitates washed once with 10 % ammonium sulphate and titrated.

Calculated	Found
49·70 mg.	48·08
30·80	30·45

These results indicate that the correction given by Folin and Schaffer (+ 3 mg. for 100 c.c. urine) does not apply to pure solutions of uric acid.

Since ammonium sulphate was found to give fairly satisfactory results with uric acid alone, this salt was used in the separation of uric acid and purine bases.

In the uric acid estimations Hopkins's method of procedure was followed, except that ammonium sulphate in 10 % strength was used, and the mixture allowed to stand overnight as in the Folin-Schaffer process, in which the same proportion of sulphate is present. The urate precipitates were washed twice with 10 % ammonium sulphate. No correction was used for the sulphate mother-liquor. For the acid mother-liquor Hopkins's correction was used (+ 1 mg. for 15 c.c.).

The purine base solutions used were prepared from urine, as described above (p. 303). The filtrates and washings obtained from the precipitates of ammonium urate and uric acid were united, and precipitated with ammoniacal silver nitrate, the concentration of silver being the same as in the control estimations upon the purine base solution. The nitrogen of the amount of uric acid reckoned to be present in the acid mother-liquor was subtracted from the result of the purine base estimation.

It will be seen that in these estimations the purine bases show no tendency to keep the uric acid in solution.

This method has been applied to urine in the following way.

10 grs. ammonium sulphate are placed in a beaker. 100 c.c. urine and 5 c.c. strong ammonia are added, and the mixture stirred till the salt is dissolved. Next day the precipitate is filtered off, the last portions being washed on to the filter with 10 % ammonium sulphate. The filtrate and washings are received in a 250 c.c. measuring flask. The uric acid estimation is completed by Hopkins's method; less than 10 c.c. of water is required for the removal of chlorides. The acid filtrate and washings are received in a measuring cylinder, and transferred to the 250 c.c. flask. To the mixture in the flask are added 10 c.c. 20 % ammonia and 10 c.c. magnesia mixture; the flask is then filled up to the mark, and the contents filtered[1]. To 240 c.c. of the filtrate 20 c.c. ammoniacal silver

[1] In methods A and B, it is important to shake the mixture thoroughly before filtering off the phosphates. Berthelot and André[19] found that ammonium magnesium phosphate was not decomposed appreciably by boiling for an hour with magnesium oxide.

Analyses of mixtures of uric acid and purine bases.

Series I.

0·2584 gr. uric acid dissolved with ammonia in 500 c.c. water.

			Found Titration+Correction	Calculated
Uric Acid.				
(1), (2)	100 c.c. uric acid solution + 13 grs. $(NH_4)_2SO_4$ + 5 c.c. ammonia	+ 25 c.c. water	51·18, 50·43	51·68 mg. uric acid
(3), (4)		+ 25 c.c. purine base solution	50·61, 51·55	
Purine Bases.				
Solution, 25 c.c.			5·60, 5·53	Mean 5·57 mg. N
Recovered from mixture with uric acid			(3) 6·44, (4) 6·30	
Subtract uric acid nitrogen in accordance with correction (0·23 mg. in each case)			(3) 5·51, (4) 5·37	

Total Purine Nitrogen.		U.A.N.	+	P.B.N.	=	T.P.N.
Calculated		17·23	+	5·57	=	22·80 mg. N
Found	(3)	16·87	+	5·51	=	22·38
	(4)	17·18	+	5·37	=	22·55

Series II.

0·2558 gr. uric acid dissolved with ammonia in 500 c.c. water

	Found Titration+Correction	Calculated
	50·20, 49·45	51·16
	50·20, 49·80	
	8·47, 8·33	Mean 8·40 mg. N
	8·61, 8·68	
Subtract 0·7 mg.	7·91	
,, 0·6 mg.	8·08	

U.A.N.	+	P.B.N.	=	T.P.N.
17·05	+	8·40	=	25·45
16·73	+	7·91	=	24·64
16·60	+	8·08	=	24·68

nitrate is added, and the mixture left overnight. The estimation is completed by the Camerer-Arnstein method[1]. The following corrections have been used: (1) +3 mg. uric acid for 100 c.c. urine, as in the Folin-Schaffer method, and (2) +1 mg. uric acid for 15 c.c. acid mother-liquor, as in Hopkins's method. To the result of the purine base estimation $\frac{1}{7}$ is added. The amount of nitrogen which was present as uric acid is subtracted, in accordance with the above corrections. The figure obtained is the amount of purine base nitrogen in 100 c.c. urine.

Parallel estimations by methods A and B have been carried out upon several samples of urine, 100 c.c. being taken for each estimation. In series 1 and 2, 25 c.c. of 50 % ammonium sulphate was used in the estimations by method B in place of the solid salt, and the correction increased accordingly (+ 3·75 mg.).

Analyses of urine.

		Uric acid mg. in 100 c.c.	Purin base nitrogen mg. in 100 c.c.	Total purine nitrogen in 100 c.c. urine					
1	A	34·88	2·26	11·63	+	2·26	=	13·89	13·75
		34·88	1·98	11·63	+	1·98	=	13·61	
	B	36·49	2·31	12·16	+	2·31	=	14·47	14·54
		36·49	2·45	12·16	+	2·45	=	14·61	
2	A	37·23	7·40	12·41	+	7·40	=	19·81	
		36·99	lost						
	B	38·57	6·77	12·86	+	6·77	=	19·63	19·77
		38·37	7·12	12·79	+	7·12	=	19·91	
3	A	34·18	3·61	11·39	+	3·61	=	15·00	14·59
		33·94	2·87	11·31	+	2·87	=	14·18	
	B	35·37	3·07	11·79	+	3·07	=	14·86	14·80
		35·30	2·97	11·77	+	2·97	=	14·74	
4	A	28·08	2·90	9·36	+	2·90	=	12·26	12·35
		27·61	3·25	9·20	+	3·25	=	12·45	
	B	26·35	3·57	8·78	+	3·57	=	12·35	12·36
		27·48	3·21	9·16	+	3·21	=	12·37	
5	A	28·08	1·63	9·36	+	1·63	=	10·99	11·03
		28·31	1·63	9·44	+	1·63	=	11·07	
	B	28·05	2·36	9·35	+	2·36	=	11·71	11·74
		28·06	2·41	9·35	+	2·41	=	11·76	
Averages of above figures:									
			A 10·81	+ 3·50	= 14·31				
			B 11·02	+ 3·62	= 14·64				
6	A	35·58	6·14	11·86	+	6·14	=	18·00	18·10
		36·51	6·02	12·17	+	6·02	=	18·19	
	B	41·19	4·75	13·73	+	4·75	=	18·48	18·37
	(H_2SO_4)	41·17	4·55	13·72	+	4·55	=	18·27	

[1] In all the estimations which have been made by methods A and B, the filtration of the silver compounds has been carried out on an unfolded 9 cm. paper without the use of the pump.

A comparison of the results obtained by the two methods shows that method B gives on an average somewhat higher figures. It had been expected that this method would give still higher figures for the purine bases, as the method was adapted to recover any purine bases precipitated by the ammonium salt, and in the experiments which were made with reference to this (p. 303) about 10 % of the bases was so precipitated. The fact that the two methods when applied to urine give average results for the purine bases differing by much less than 10 %, may be due to the following reasons.

(1) The purine bases of urine are probably precipitated by salts more readily after they have been separated from urine, as is the case with uric acid (p. 305). The experiments described above (p. 303) which were carried out upon purified bases, and which led to the employment of method B, may therefore indicate a degree of error in method A which is not present in analyses of urine. Moreover, the proportion of the purine base or bases in urine precipitable by ammonium sulphate will not be constant, and this proportion may have been high in the urine from which the purified bases were obtained.

(2) During the first two filtrations in method A there is considerable opportunity for evaporation, which will tend to raise the figure obtained for the purine bases. In B, evaporation could affect the result only during the final removal of the phosphates, which is effected very rapidly.

(3) Method B requires the higher correction for unprecipitated uric acid. Such corrections do not pretend to a high degree of accuracy, and their error will have a marked effect in one or other direction upon the figures obtained for the small proportion of purine bases.

In the analyses of urine given on p. 307 it is seen that, in each of the six series, with the exception of the first, the method giving the higher result for the uric acid gives the lower result for the purine bases. This is especially evident in series 6; in this case, in method B, sulphuric acid (4 drops) was used in place of hydrochloric acid for the decomposition of the ammonium urate. Hence the figures obtained by the two methods for the total purines agree together more closely than do those for either the uric acid nitrogen or purine base nitrogen. The explanation of this would appear to be that the amounts of unprecipitated uric acid are variable, and these amounts will be recovered in the purine base estimation.

The amount of purine base nitrogen in 100 c.c. of urine being so small, the estimation of this amount is naturally subject to considerable error. This is seen in the discrepancy between some of the duplicate results given on p. 307.

The error due to the process of analysis, as distinct from that due to the use of somewhat arbitrary corrections, may of course be lessened by using larger amounts. Several portions of 100 c.c. of urine may be prepared in the manner described above; two of these are used for the estimation of uric acid; in the others the amount of uric acid obtained is not determined, but all the filtrates are used for the estimation of purine bases.

The methods put forward here have an advantage in that the estimations of uric acid and purine bases are carried out upon the same sample of urine, whereby an economy is effected which is of importance in experiments in which the urine is analysed several times daily. Method A has advantages over method B in that the analyses are completed in a shorter time, and the process of uric acid estimation is simpler.

Summary.

Uric acid, when treated by the Camerer-Arnstein method for the estimation of the total purines of urine, loses nitrogen in the form of ammonia. The formation of ammonia occurs when the precipitate of silver-magnesium urate is boiled.

The purine bases which were examined were found not to undergo this loss.

The Camerer-Arnstein method can be used for the estimation of the purine bases in urine from which the uric acid has been removed, but is subject to considerable error when small quantities only of urine are dealt with.

In conclusion, I wish to acknowledge my indebtedness to Dr Leathes and Dr Aders Plimmer for advice in carrying out this work, which was done during the tenure of a Research Scholarship of the British Medical Association.

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THE INFLUENCE OF CARBOHYDRATES AND FATS ON PROTEIN METABOLISM.

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THERE has always been a difficulty in drawing conclusions as to the effects of certain factors on endogenous metabolism since, unless in fasting, the excretory products may have come from either an exogenous or endogenous source, or from both of these.

Folin[1] in his paper on protein metabolism has stated that there are certain excretory substances characteristic of endogenous protein metabolism and others of exogenous. The substances which he classed in the first group were creatinine and neutral sulphur, and in the second urea and inorganic sulphates.

Experience has shown that it is very difficult to alter the daily output of creatinine to any considerable extent and yet if it were possible this would be the ideal product through which to investigate endogenous metabolism, as the method of quantitative estimation is so simple.

In 1907 I[2] found, as did also Benedict[3] about the same time, that creatine was a constant excretory product during inanition. I further noted that as soon as food was supplied the creatine practically disappeared. Here then was a product of pure endogenous origin the study of which might give some insight into the course of protein metabolism in the animal body. From the nature of the food given after the fast this diminution in the output of the creatine must have been due either to the influence of the carbohydrate and fat combined or to one of these alone. From some preliminary experiments carried out at the end of 1907 and from analogy with certain other feeding experiments which will be referred to later I came to the conclusion that the "active" part of the food was the carbohydrate.

In order to test thoroughly this supposition a series of experiments were designed, where, after a period of fasting in order to bring about

the production of creatine as an excretory product, the food given would consist solely of one class of food stuffs—carbohydrates or fats.

I.

Each experiment from I to IV began with a complete fast of 40 hours duration which always brought about an output of some 150 mgrms of creatine. A fast of 60 hours was tried but without any corresponding gain in the output. This fast was followed by the ingestion of (1) carbohydrate food which consisted in two experiments of tapioca boiled with water, or made into small cakes with water, and eaten with a small quantity of honey. In another experiment the carbohydrate was in the form of banana meal made into small cakes with water and eaten with honey. (2) Fats. An attempt was made to live on pure butter and nothing else, but after two meals this food was abandoned on account of the nausea produced. Centrifugalised double cream (fat content 50—55 %) was substituted. This contained very little nitrogen and was practically free from carbohydrate.

I myself was the subject of experiment in experiments I, II, III and V.

The methods of analysis employed were total nitrogen—Kjeldahl, urea—Folin or Morner-Folin, ammonia—Folin-Schaffer, uric acid—Hopkins-Folin, creatinine and creatine—Folin. The amount of carbohydrate in the fæces by Liebermann's method and the amount of fat by Soxhlet's method. The urine of the first 16 hours of fasting was always discarded; therefore the urine of the period marked fast in the tables was a 24 hours sample.

TABLE I.

Day of Exp.	Nitrogen in grms.						Per cent. of total nitrogen					
	Total	Urea	Ammonia	Uric Acid	Creatinine	Creatine	Urea	Ammonia	Uric Acid	Creatinine	Creatine	Diet
1	5·95	4·46	·396	·075	·445	·067	75·0	6·6	1·2	7·5	1·1	Fast ($-H_2O$).
2	7·85	6·22	·666	·146	·525	·021	79·2	8·4	1·8	6·7	0·2	Carbohydrate.
3	4·91	3·50	·493	·088	·509	·001	71·3	10·04	1·7	10·3	0·00	,,
4	9·15	—	—	—	·666	·000	—	—	—	7·2	0·00	Mixed.

Carbohydrate diet.

Tapioca	=454 grms.	Fæces = Carbohydrate.
Sugar	=114 ,,	19·0 grms.
Honey	=227 ,,	4·5 ,,
Cornflour	= 85 ,,	

Calorie intake = 40 Cal. per kilo.

It will be noted here that instead of the usual fall in the output of nitrogen which is observed after the ingestion of carbohydrate there is a very distinct rise in all the products excreted with the exception of the creatine. This rise I believe is due to the washing out of the tissues as the fast was absolute, *i.e.* without either food or water. Naturally under these conditions the flushing out of the tissues during the fasting period was not thorough and it was to be expected that when the carbohydrate was begun the following day with a fair amount of fluid (about 500 c.c. of water was taken each day) there would be some washing out. In spite of this there is a very distinct fall in the output of creatine nitrogen from 67 mg. to 21 mg. The second day of the carbohydrate diet shows the usual effect, a fall in the total nitrogen below the starving total nitrogen with a similar result in the case of the urea. The ammonia has fallen below the amount excreted on the first day but is still above the fasting level. The uric acid is almost back to fasting level and the total creatinine output, *i.e.* preformed creatinine plus creatine, is also almost identical with that excreted during the fasting period (see Table VI). The preformed creatinine is considerably higher than on the day of the fast but the output of creatine has to all intents and purposes ceased. On the resumption of the ordinary mixed diet the mere trace of creatine present completely disappeared.

In order to test whether the increase of nitrogen excreted after the commencement of the carbohydrate food was due to simple flushing out another experiment in every way comparable with the first was carried out except that during the fast water to the extent of 500 c.c. was taken.

TABLE II.

Day of Exp.	Nitrogen in grms.						Per cent. of total nitrogen					Diet
	Total	Urea	Ammonia	Uric Acid	Creatinine	Creatine	Urea	Ammonia	Uric Acid	Creatinine	Creatine	
1	6·80	5·33	·423	·076	·433	·057	78·3	6·2	1·1	6·3	0·9	Fast ($+H_2O$).
2	6·85	5·61	·521	·089	·438	·007	81·9	7·6	1·3	6·4	0·1	Carbohydrate.
3	7·14	—	—	—	·535	·005	—	—	—	7·4	0·1	Mixed.

Here it will be noted that the taking of the food does not cause any appreciable rise in the output of nitrogen—a slight fall had indeed been looked for. The outputs of ammonia and of urea have risen somewhat but along with this there is a remarkably low excretion of uric acid. The total creatinine excreted is lower than on the first day of the fast

although the preformed creatinine is somewhat higher. Here again under the influence of the carbohydrate feeding the output of creatine nitrogen has fallen from 57 mg. to 7 mg. With the resumption of the mixed diet there is a marked rise in the output of preformed creatinine and a very small output of creatine.

As the results of carbohydrate feeding were so definite it was next determined to try the effect of feeding with fat.

TABLE III.

Day of Exp.	Nitrogen in grms. Total	Urea	Ammonia	Uric Acid	Creatinine	Creatine	Per cent. of total nitrogen Urea	Ammonia	Uric Acid	Creatinine	Creatine	Diet
1	7·62	6·33	·396	·082	·424	·051	83·2	5·2	1·1	5·5	0·7	Fast ($+H_2O$).
2	11·67	8·54	·564	·043	·370	·141	73·1	4·8	0·36	3·1	1·2	Fat.
3	9·09	7·43	·945	·038	·353	·122	81·7	10·3	0·42	3·8	1·4	,,
4	15·44	12·41	1·60	·128	·372	·118	80·3	10·3	0·82	2·4	0·7	Fat-Protein (sugar free).
5	17·04	13·04	1·52	·193	·420	·130	76·5	8·8	1·1	2·4	0·8	,, ,,
6	14·28	—	—	—	·497	·085	—	—	—	3·4	0·7	Mixed, little carbohydrate.
8	—	—	—	—	·534	·000	—	—	—	—	0·0	Mixed, ordinary.

Fat diet. 570 grms. Cream (55 % fat) = 312 grms. fat. 40 Cal. per kilo.
Fæces = 18 grms. fat. [The fat on the whole was well utilised. Only very slight diarrhœa caused. The same is true of Exp. IV done on Dr Graham Brown.]

Fat-Protein diet.

Casein bread (Carbohydrate free)		= 170	grms.
Cheese	... ...	= 43	,,
Butter	... ...	= 128	,,
Eggs ...	... ...	= 12	,,

39 Cal. per kilo.

Here again as in previous fasts there was an output of creatine. On the first day of the fat feeding, although the usual 500 c.c. of water had been taken during the fasting period, there was a very definite rise in the output of total nitrogen. Along with this rise however there is a fall in the percentage output of urea and of ammonia but a rise in the absolute amount excreted. In the case of the uric acid and the creatinine there is both a percentage and an absolute fall. Creatine in contradistinction to the experiments with the carbohydrate shows a very marked rise both in the absolute and in the percentage amount excreted. This high output of creatine is continued on the second day of the fat

feeding. The output of total nitrogen on the other hand has fallen on this day but there is a percentage rise in the output of urea. The ammonia, as was to be expected remembering the marked acidosis which follows pure fat feeding when all carbohydrate is absent, has risen very considerably, forming over 10 % of the nitrogen excreted. The preformed creatinine output has fallen still further, whereas the total creatinine output (see Table VI) which rose on the first day of the fat diet has on the second returned exactly to the fasting level.

When the diet was changed by the addition of a large amount of protein but still kept strictly carbohydrate free there is no great alteration in the percentage amount of the products excreted. Naturally the total nitrogen has risen very considerably as the result of the increased nitrogen intake. The percentage output of urea is hardly altered and the ammonia on the first day of the new diet remains at its old high figure of some 10 % of the total nitrogen, but on the second day there is a slight fall. As regards the uric acid there is a steady rise during the two days (the diet is purine free) both in absolute and percentage amount well above the starvation figure. This is quite in agreement with the observation of Maurel[10] that alterations of the nitrogen content of a diet bring about alterations in the uric acid output especially during the condition of nitrogen hunger. Folin on the other hand does not believe that variations in the protein intake affect the uric acid output. In the case of the preformed creatinine there is quite a distinct rise in absolute amount excreted and this rise is continued on the second day of the fat protein diet, reaching then the fasting level. Creatine on the other hand is still present in large amount: a very slight drop occurs on the first day but this is followed by a well-defined rise on the second day. As regards the total creatinine (see Table VI) it will be noted that the output of the second day exceeds easily the output of any other day of the experiment. This is hardly in agreement with the statement of Folin that the output of creatinine is quite uninfluenced by alterations in the diet: this disagreement holds equally true if the amount of preformed creatinine excreted be considered alone.

Finally a mixed diet was given in which there was only a small amount of carbohydrate with the result that there was a still further rise in the output of preformed creatinine and a distinct although not a very great fall in the output of creatine. Two days later on an ordinary mixed diet an examination of the urine showed that there was an absolute increase in the amount of preformed creatinine excreted and

an absence of any creatine. The total amount excreted on this day is less than the total amount excreted on the first day of the modified mixed diet following the fat protein diet.

As I desired confirmation on another subject of this effect of a fat diet Dr T. Graham Brown very kindly put himself at my disposal.

The diet in this experiment as in the last consisted of cream and was taken to the extent, after making allowance for the loss by the fæces, of 48 Cal. per kilo.

TABLE IV.

Day of Exp.	Nitrogen in grms.			Per cent. of total nitrogen		Diet
	Total	Creatinine	Creatine	Creatinine	Creatine	
1	7·16	·377	·042	5·2	0·6	Fast (+H_2O).
2	10·74	·324	·102	3·0	0·9	Fat.
3	17·05	·526	·072	3·1	0·4	Mixed.

Here again it will be noted that there is a very definite rise in the output of the creatine on the fat diet which diminishes when a mixed diet rather poor in carbohydrate but rich in protein is taken. There is a definite fall in the amount of preformed creatinine excreted but a rise in the amount of total creatinine. The amount of preformed creatinine and of total creatinine excreted is very markedly increased with the resumption of the mixed diet. This experiment also demonstrates the rise in the output of total nitrogen which follows the ingestion of a nitrogen poor fat diet.

Another experiment was carried out where the feeding period was not preceded by a fasting period. In this case the carbohydrate diet was continued for five days followed by a fat period of two days duration.

I. Carbohydrate diet.

Banana meal[1]	= 454 grms.	
Honey	= 230	,,

II. Fat diet.

Butter	= 65	,,
Cream (55 %)	= 340	,,

Calorie intake = in I 32 Cal. per kilo; in II 35 Cal. per kilo. (In both allowance for the loss by the fæces is made.)

[1] Banana meal dried at 100° C. Nitrogen = 0·64 %, Sugar = 83·3 %, Fat = trace. Honey Nitrogen = 0·23 %, ,, Sugar = 75 %.

TABLE V.

Day of Exp.	Nitrogen in grms. Total	Urea	Ammonia	Uric Acid	Creatinine	Creatine	Per cent. of total nitrogen Urea	Ammonia	Uric Acid	Creatinine	Creatine	Diet
1	6·79	4·61	·375	·123	·478	·004	67·8	5·5	1·8	7·04	·06	Carbohydrate.
2	6·40	4·65	·134	·173	·460	·015	72·7	2·1	2·7	7·18	·24	,,
3	4·77	3·21	·132	·146	·413	·007	67·2	2·7	3·1	8·65	·15	,,
4	4·79	3·17	·121	·152	·450	·004	66·2	2·5	3·1	9·37	·08	,,
5	4·39	3·31	·104	·125	·436	·000	75·3	2·3	2·8	9·93	·00	,,
6	4·83	3·76	·238	·157	·400	·019	77·8	4·9	3·2	8·28	·39	Fat.
7	8·13	6·64	·527	·088	·347	·091	81·6	6·4	1·1	4·26	1·12	,,
8	16·21	13·45	1·13	·551	·477	·001	83·0	6·9	3·4	2·94	0·0	Mixed.

The total nitrogen in this experiment during the carbohydrate period shows a very definite and steady fall in the amount excreted to be followed by just as well a defined rise in output on the fat diet. This is in absolute agreement with the observations of Landergren who was the first and as far as I am aware the only one to draw attention to the curious curve of the nitrogen excretion as the result of a carbohydrate rich followed by a fat rich but in both cases nitrogen poor diet. The percentage amount of urea excreted falls as low as 66·2 % on the carbohydrate diet but is about 80 % on the fat. The absolute amount of ammonia excreted falls steadily during the carbohydrate period although the percentage amount remains practically constant. With the fat diet there is a rise in the output of ammonia both in absolute and percentage amount as was to be expected from the acidosis which follows the ingestion of fat. On each of the two days of the fat diet the acetone and diacetic acid reactions were extremely well marked. On the carbohydrate diet the output of uric acid is to be regarded as very regular whereas on the second day of the fat diet quite a marked fall in the output takes place. This was followed by a very decided rise on the resumption of the mixed diet due to the ingestion of exogenous purine. The preformed creatinine on the carbohydrate diet in absolute amount excreted remained remarkably constant, with the result that in percentage amount of the total nitrogen there was a steady rise. With the fat diet there was at once a fall in the amount excreted followed by a rise when the mixed diet was resumed. As regards the creatine output it will be noted that throughout the carbohydrate period there is a trace present except on the last day of the period. The maximum output is 15 mg. on the second day of the experiment. This small output may have been due to undernutrition as the banana

meal was not on the whole very well utilised. This is supported by the fact that during this experiment there was some loss of weight. During the two days of fat feeding there is a well-marked rise in the output of creatine: 19 mgs. on the first day rising to 91 mgs. on the second. With the resumption of the mixed diet the creatine present practically disappears.

Brief reference may now be made to the variation in amount of the different nitrogenous excretory products estimated, viewed in the light of all the experiments done.

Urea. As Folin (*loc. cit.*) showed, when a subject is put on a low nitrogen diet there is at once a fall in the percentage output of urea. This fall is very well marked in the case of the feeding with the carbohydrate as in Table V where the urea output has fallen to 66·2 % of the total nitrogen on the fourth day of the experiment. This fall is not so marked in those experiments where the carbohydrate was preceded by a fast. Of course it must be taken into account that in these fasting experiments the duration of carbohydrate feeding was short. In the case of the fat diet although again nitrogen free no such definite fall in the percentage output of urea is noted. On the second day of fat feeding in both III and V the urea forms a substantial proportion of the total nitrogen excreted (81·6 %).

Folin from his experiments with the nitrogen low and nitrogen high dietaries founded his theory of the dual protein metabolisms, the so-called exogenous and endogenous metabolisms. As principal excretory product of exogenous metabolism he puts down urea, basing his conclusions on the variations observed in its output during high and low nitrogen feeding. Folin however it must be remembered never states that no urea comes from endogenous sources. The results of my experiments would however seem to throw some objections in the way of the complete acceptance of the two forms of metabolism being absolutely distinct one from another. Here from a practically purely endogenous source we have a percentage excretion of urea not far short of that yielded by a protein rich mixed diet (cf. Noël Paton[m]). The present series of experiments was not however designed for the elucidation of this problem, and as the experiments done are too few in number to settle this particular point the question will be left for the present.

Ammonia. There is nothing exceptional in the excretion of this body. As was to be expected there was a marked rise in the output of ammonia during the fat periods due to the increased acidosis. The

amount of acetone and diacetic acid excreted during the fat period was, judging solely from qualitative tests, very large. On the carbohydrate dietary the output during the period of feeding without previous fasting (Table V) was comparatively low and steady. In the experiment where fasting preceded the carbohydrate dietary I and II the output of ammonia rises markedly, in experiment I equalling indeed the output of the fat period. It is to be noted here that in this experiment although the acidity of the urine was not estimated it was probably high as there was a slight degree of alimentary glycosuria, therefore also probably some partially combusted acid carbohydrate compounds were excreted.

Uric Acid. The output on the carbohydrate diet without previous fasting (V) was remarkably steady and high if it be compared with the other experiments. On the fat diet it would seem that the output is lower. One curious point to which attention has already been drawn is that on the protein fat diet—a purine free diet—there is a rise in the output of the uric acid.

Creatinine and Creatine. The most interesting observation in the above experiments is that the excretion of creatine which is induced by starvation tends to disappear on a carbohydrate but to increase in amount excreted on the fat diet.

TABLE VI.

Exp.	Preformed creatinine in grms.	Total creatinine in grms.	Creatine in grms.	Diet
I	1·200	1·382	0·182	Fast.
	1·416	1·474	0·058	Carbohydrate.
	1·373	1·375	0·002	,,
II	1·168	1·321	0·153	Fast.
	1·182	1·200	0·018	Carbohydrate.
III	1·144	1·282	0·138	Fast.
	0·998	1·378	0·380	Fat.
	0·952	1·280	0·328	,,
	1·004	1·321	0·317	Fat-Protein.
	1·134	1·483	0·349	,,
IV	1·018	1·131	0·113	Fast.
	0·874	1·150	0·276	Fat.
V	1·240	1·280	0·040	Carbohydrate.
	1·112	1·136	0·024	,,
	1·212	1·224	0·012	,,
	1·169	1·159	0·000	,,
	1·080	1·128	0·048	Fat.
	0·936	1·182	0·246	,,

Creatinine (preformed). On the carbohydrate diet the output in the experiment without fasting (V) and in II remains fairly constant, but in II there is a tendency for the output to increase. Viewed in the light of total creatinine the output on the carbohydrate diet may be regarded as fairly constant throughout the different experiments. On the fat diet there is in each case a steady decrease in the amount of preformed creatinine excreted whereas when viewed in terms of total creatinine no such great diminution is to be noted. The average output of total creatinine (calculated from Table VI) on the carbohydrate diet is 1·27 grm. whereas on the fat diet it is 1·24 grm. It would seem then that there is after all some close connection between the creatine and the creatinine, almost amounting to the definite proof of the origin of creatinine from creatine. At any rate with the appearance of creatine in any amount as in the fat experiments above detailed there is a corresponding fall in the output of creatinine.

II.

As Bayliss and Starling[5] say, it is now practically certain that no longer is one justified in valuing a diet simply on its calorific value and its content of fat, carbohydrate and protein. Hopkins and Willcock[7] have also drawn attention to the same point as the result of their feeding experiments with zein and have suggested that certain of the substances taken in the food might be utilised without directly contributing to tissue formation or structural maintenance. Further, Folin[8] in his work has suggested the probability of a special tissue metabolism which would probably entail in turn the necessity of special food products. Again too in the question of the amount of protein—nitrogen—which must be consumed our ideas are undergoing a change. All workers now are at one on the point that the body can exist on a much lower intake of nitrogen-containing food per diem than was formerly believed to be possible. Chittenden[9], Siven[10], Landergren[11], Folin (*l.c.*) and others have all given demonstrations of this. The low intake of nitrogen is not the point. The nitrogen-containing food consumed must contain a sufficient amount of the particular nitrogenous substances required for repair. Thus if the body demands x amount of some particular substance for its reconstruction the food consumed must contain that amount although in getting it x^{10} of this and x^{50} of that other nitrogen rich substance, not required for the immediate process of repair, are consumed at the same time.

Take for example such feeding experiments as those of Kaufmann[13] and of Murlin[35] with gelatine where, in spite of a very large intake of nitrogen, equilibrium could not be maintained and at the same time note the improvement in the conditions when small amounts of certain aromatic bodies, normally absent from gelatine, are added. Or again consider the feeding experiments of Hopkins and Willcock (*l.c.*) with zein where improvement followed the addition of quite small amounts of tryptophane to the diet. The recent work of Michaud[36], as yet unconfirmed, is one of the most important as a demonstration of how on feeding with a food perfectly suited to a given animal's needs—in his case feeding a dog with dog flesh—the intake of nitrogenous bodies can be reduced to a minimum.

Formerly it was generally held that protein tissue alone contributed to the repair of waste in the organism and that the carbohydrates and fats were concerned in the supply of energy. Now however it is admitted that the carbohydrates do something more than supply the energy, but the nature of this something is not very definitely understood. It has been frequently observed that when carbohydrates are given in large amount the intake of nitrogen can be reduced and at the same time the excretion of nitrogen lowered, in other words the carbohydrate acts as a protein sparer as it is generally called. The same power has been ascribed to fats by some but denied by others. Carl Voit[15] admitted that the carbohydrates were more active in this respect than the fats although in his opinion neither equalled protein or gelatine. Others again like Frentzel and Reach[16] hold that fats and carbohydrates act in an equally economical fashion. Zuntz[37] found, by means of respiration experiments, that fats and carbohydrates can be utilised in equal degree during work for the saving of protein. He states further that a starving animal made to work obtains practically all its energy from fat sources. Lusk[18] found that carbohydrates have a greater power of conserving protein than fat either given in the diet or obtained from the body's supply. Chauveau[19] maintained that carbohydrate was the important body and that fat could only act after it had been converted in the liver into carbohydrate. He supported his own experiments by reference to the conversion of fat into carbohydrate in hibernating animals. The low respiratory quotient which at certain times has been observed in these animals by many workers, Pembrey[38], Weinland[39], and others, has been explained as indicating the combustion of the fat with the formation of sugar and the storing of the same in the muscles and liver as glycogen. The interesting point

about this in the present connection is that the formation of carbohydrate would seem to take place just about the time the animal was going to awake and indulge in more active muscular exercise, i.e. increase the daily breakdown of tissue protein. The formation of carbohydrates from fatty bodies in seeds, or at any rate the observation that with the rise in the carbohydrate content there is a fall in the fat content of germinating seeds, must not be forgotten (Leclerc du Gablon[22], Mazé[23], and others). Further Pflüger[24] produces evidence which strongly suggests the possible conversion of fat to sugar.

The present experiments and those of Landergren (*l.c.*) however show that under our conditions—nitrogen hunger—the power of carbohydrate and fat to spare the breakdown of tissue protein is by no means equal. I may quote further the experiments of Kayser[25] who found that, even with a diet rich in protein, when carbohydrate was removed and the calorie value of the food made up with fat there was an immediate increase in the breakdown of the protein of the tissues. Landergren found that if he gave a diet made up of pure carbohydrate, practically nitrogen free, the output of total nitrogen steadily fell, but when the carbohydrate was replaced by a diet consisting solely of fat, again nitrogen free, the nitrogen output steadily rose in amount.

Landergren however does not utilise his figures for the discussion of a probable synthesis but develops a hypothesis as to the varying nature of the nitrogen present in the protein molecule. Table V in the present paper also demonstrates this rise in the output of total nitrogen after changing from a carbohydrate diet to a fat diet both containing very small amounts of nitrogen. Further in my experiments the behaviour of the creatinine-creatine output shows another difference between the effect of feeding with fat and with carbohydrate, probably connected however with the factor which influences the total nitrogen output.

Landergren drew attention to two other points in his paper which may be mentioned here. He showed that there is no relation between the so-called isodynamic ratios of fat and carbohydrate and this power of sparing tissue protein. He makes out that in this connection the carbohydrate is twice as active as an isodynamic amount of fat. Again he showed that the increased output of total nitrogen on a fat diet does not go on to an unlimited extent but that, as a rule, the maximun output is reached on the third day of fat feeding.

The effect of fat and carbohydrate diets has been most extensively studied in connection with muscle at work and not at rest as in the

present instance. As however resting muscle may be regarded as undergoing the same changes only to a less degree as muscle doing voluntary work the conditions may be looked on as identical. Work probably merely increases the metabolism in the true sense of the word—the exchange of material.

Practically every investigator who has worked at this problem of the utilisation of material during work has found that the work done leads to no or only a very slight increase in the output of nitrogen, provided always the supply of food particularly carbohydrate and of oxygen be sufficient (Hirschfeld[96], Zuntz[97], Paton[98], Voit (*l.c.*) and others).

It is a fundamental mechanical law that every piece of machinery wears with work, the parts where there is the greatest friction and use more rapidly than the rest. Have we then to look on muscular tissue, the part most involved both at rest and during work, as being beyond wear and tear, a piece of mechanism on a level with the practically unwearable jewelled bearing? Far from it, because it is very readily demonstrable that if the work be carried on under unfavourable conditions we get immediate evidence of the utilisation of protein tissue (wear and tear of muscle?) in the form of an increased nitrogen output. What then is the course of events provided the conditions be favourable? Why is there no appearance or only a very small one of nitrogen as the result of work?

The small increased output of nitrogen from work may be explained in two ways at least:

(i) That the actual wastage of protein tissue is small in amount; that the protein tissue as it is broken down divides into two parts, a nitrogen free part which is utilised for the energy needs, and a nitrogen containing part which is resynthesised.

(ii) That the work increases the breakdown of protein tissue, but instead of this nitrogen appearing in the urine as an increased excretion there is a similar amount taken from the food nitrogen to replace the nitrogen set free by the work done. The result would be that no practical difference in the nitrogen excretion was to be observed although there had been an alteration in its source of origin, more coming from the endogenous source and less from the exogenous.

The first hypothesis presupposes that the actual nitrogen requirement of the body is low and that it could exist on a food of sufficient calorie value made up mainly of nitrogen free substances. Siven (*l.c.*) and others have shown that such is the case. The body cannot exist indefinitely in this condition, unless the source of the nitrogen

supply be a perfect source, as there is a constant, although perhaps small, loss which has to be made good from some outside source. As I have already noted the low intake of nitrogen is not the point: it is not the quantity but the quality of the nitrogenous food which is of importance.

The second hypothesis presupposes that the slight excess of nitrogen which appears as the result of work must come from the breakdown of protein tissue and therefore if the hypothesis be right then all the nitrogen excreted must have come from broken down protein tissue, the result of the work. There would be too little nitrogen supplied by the food to make up the nitrogen loss. This hypothesis also would require the acceptance of the statement of Pflüger[20] that the need of food is satisfied first by protein, otherwise in those experiments where the intake of protein was high there should have been no appearance of excess nitrogen output. The work of Krummacher[30] shows that even with a very large intake of protein the increased output of nitrogen after work still takes place. The same worker also shows that the possible energy calculated from the nitrogen excreted does not equal the energy expended in the work. Frentzel[21] likewise demonstrated in his experiments that if not only the excess of nitrogen excreted be held as coming from protein utilised during work but that the total amount of nitrogen excreted on the day of the work be regarded as from this source not even then was there sufficient material combusted to furnish the energy expended. The energy calculated from the amount of nitrogen excreted in one experiment equalled only about two-thirds of the work done.

Both these latter observers however apparently believed that the amount of nitrogen excreted represents exactly the amount of protein tissue metabolised in the body. Such a belief is not now so generally held. Take for example the case of inanition. Here it must be admitted that part at least of the nitrogen liberated by the breakdown of protein tissue within the body is resynthesised, as certain tissues and organs retain practically their original weight up to the last (Voit, Chossat).

The idea of a resynthesis taking place within the tissues particularly the muscles is not a new one. Hermann[22] 40 years ago put forward the hypothesis that the protein in all probability was broken down into a nitrogen containing part which was reutilised in some way and a nitrogen free part which was used. He believed further that an increase in the output of nitrogen took place only when the work done was very

prolonged and severe, when an actual destruction of muscle fibres was brought about. Pflüger[33] has offered the hypothesis that all metabolism is a partial breakdown with a subsequent regeneration of the living protein molecule. Verworn[34] supports also such a view holding it conceivable that under certain circumstances regeneration of the nitrogenous residues can take place at the expense of the food stuffs and oxygen. As he says "such economy with the costly nitrogen would be wholly in accord with the methods of the organic household."

To elaborate this hypothesis further was the object of the present investigation. In order to get some insight into the metabolic changes it was considered necessary to attempt the study of one product of protein breakdown and preferably a product which was not one of the ordinary end products. Creatine was the substance chosen as it never appears in the urine as an excretory product under normal conditions (Folin, *l.c.*) but which can be readily caused to appear having as its chief source of origin the muscle tissue.

As has already been pointed out the ingestion of a diet consisting of pure carbohydrate practically nitrogen free leads to a very marked fall in the output of creatine present as the result of fasting and at the same time as Landergren and others have noted to a fall in the output of total nitrogen. On the other hand a diet again practically nitrogen free but consisting of fat does not bring about the fall in the output either of total nitrogen or of creatine during two days although there is reason to believe that after three or four days such a fall in the case of the total nitrogen at least begins. It is further to be noted in this connection in Exp. III the output of creatine is lower on the second day of the fat diet than on the first, which points to a reduction in the breakdown of protein.

It is believed that the above experiments may be regarded as giving ground for the acceptance of the hypothesis that a resynthesis takes place in the tissues (muscle?) and that the greater part of the muscle protein tissue nitrogen which is set free as the result of work does not appear in the urine but is reutilised. Further that the food stuff which plays the most important *rôle* in this resynthesis is carbohydrate. Fats apparently must undergo some change which requires time to produce—the fall which follows the preliminary rise in the output of total nitrogen on a fat diet does not take place until the second or third day. This change which fats undergo may be the conversion into carbohydrates advanced by Chauveau and supported by the observations on hibernating animals, germinating seeds, etc.

The following diagrams which are based on one which appeared in a paper by Professor Noël Paton (*l.c.*) and which he has kindly permitted me to use will perhaps aid in making the position clear. It is presumed that N_n is the amount of nitrogen set free by protein breakdown in a muscle. In diagram I the supply of oxygen and carbohydrate has been sufficient so that N_s is resynthesised and N_i is passed on for excretion with the CO_2 the product of the combustion of nitrogen free material. In II the supply of carbohydrate has been deficient, with the result that there is a resultant loss in the nitrogen set free so that only say N_s is resynthesised and N_4 is passed out as waste.

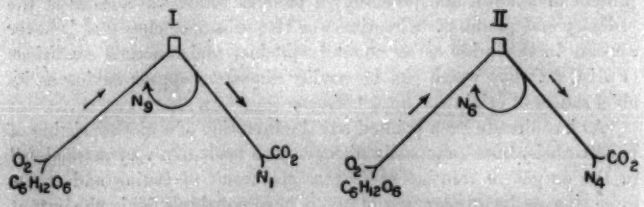

Fig. 1. I. Carbohydrate adequate. II. Carbohydrate inadequate.

That carbohydrates play an all important part in the utilisation of protein given in the diet has been repeatedly demonstrated. The best example of this is in the feeding of animals with biuret free digest products where unless there is an abundant supply of carbohydrates there is no retention of nitrogen. The experiments of Lesser[36] which followed the publication of Loewi's[34] epoch making work are a most excellent demonstration of this very point. Lesser was unable to confirm Loewi's results but an examination of his protocols shows that in his experiments he omitted carbohydrate altogether from his diets, using fats and protein digest products alone. Lüthje[37] in an elaborate paper has proved beyond any manner of doubt the absolute importance of the carbohydrate moiety in the utilisation of protein. He suggests that some form of amino sugar is first of all elaborated.

Again we have the support of the botanical physiologists (Hansteen[38], Iwanoff[39] and others) who have time and again demonstrated that the presence of carbohydrate is absolutely essential before protein synthesis takes place.

Then we may cite the rise in the output of total nitrogen which is

frequently observed in cases of diabetes when the sugar instead of being utilised in the body is excreted. Further during starvation the sugar content of the blood keeps high, a probable provision for the reutilisation of the products of protein disintegration formed during this condition.

Except for the suggestion of Pflüger (*l.c.*) that the nitrogen containing part of the broken down protein molecule combines with "alcohol radicles" to form new protein no experimental work that I am aware of on this synthesis hypothesis exists. It is of course well known that aldehydes will form compounds with many different substances containing nitrogen. Take for example the most simple of all these compounds, the union of aldehyde with ammonia to form aldehyde ammonia. Not only will a simple aldehyde like acetaldehyde unite with ammonia but Lobry de Bruyn [40] has formed compounds from sugars and ammonia. Again Morrell and Bellars [41] have combined guanidin with dextrose and lævulose and Wolff [42] found that amidoguanidin would form compounds either with a simple aldehyde or with sugar. Schoorl [43] has formed a compound of glucose and urea and Jaffe [44] found that creatine (and also creatinine) could unite to form a compound with formaldehyde. Tryptophane can also combine with aldehydes. The formation of methylimidazol which Knoop and Windaus [45] observed from glucose in the presence of ammonia and zinc oxide is also of interest in this connection as this imidazol ring occurs in histidine, creatinine and purine. Sorensen [46] has shown that the simple monamino acids can unite with formaldehyde. Spiegel [47] has suggested that certain peptide groups in the protein molecule are combined by means of carbon atoms and has attempted to get a synthesis of protein from the decomposition products of the protein molecule by means of formaldehyde. He states that he has obtained products which suggest a synthetic change.

In the body if some similar synthesis does take place it is much more likely to occur between the nitrogen containing rest of the decomposed protein molecule and one of the lower aldehydes resulting from carbohydrate decomposition than with the complete carbohydrate molecule. One product which can readily be obtained from the decomposition of glucose *in vitro* is lactic acid and this same acid is also obtained from isolated muscle as the result of certain conditions for example work. Further if the supply of oxygen is deficient as in the experiments of Araki [47] lactic acid appears in the urine; it also appears when excessive exercise is taken (Ryffel [48]). Now the steps of the formation of lactic acid from glucose as given for instance by Wohl [49]

show the production of two actively reacting aldehydes before the final lactic acid is reached, namely glyceric aldehyde and pyruvic aldehyde (methyl glyoxal), either of which might unite with the nitrogen rest. (*In vitro* experiments with both these substances are at present being carried out.) Or again it is quite possible that the necessary aldehyde may be formed from the lactic acid by further decomposition.

Fletcher and Hopkins[30] in their paper on the production of lactic acid in muscle state (p. 295) that "it is conceivable that the disappearance of lactic acid during recovery from fatigue does not involve its oxidative removal in the form of carbonic acid and water but may be due to the occurrence of reconstructive processes." Leathes[31] has also suggested that lactic acid may not be an end product but an intermediate one in some synthetic or transformative change.

The hypothesis here put forward could also explain the curious observations of Mellanby[32] and of Van Hoogenhuyze and Verploegh[33] that in cancer of the liver there is much creatine present in the urine, as the liver is the organ most deeply concerned in one stage at least of the carbohydrate metabolism. The glycogen storing capacity or the glycolytic power of the liver being interfered with there would no longer be a proper supply of sugar available, with the result that faulty and incomplete synthesis would take place. This same hypothesis might also explain to a large extent the appearance of such large quantities of nitrogen in the urine after phosphorous poisoning, a condition where the liver is again principally involved.

Conclusions.

With a carbohydrate diet, practically nitrogen and fat free, there is a fall in the output of urinary nitrogen.

With a fat diet, practically nitrogen and carbohydrate free, there is a marked rise in the output of urinary nitrogen.

During starvation creatine is constantly present in the urine.

The output of creatine induced by fasting at once falls when the diet consists of carbohydrate, whereas with the fat diet the amount excreted increases.

The amount of creatine excreted during the fat period is not markedly reduced by the addition of protein food (carbohydrate free) to the diet.

The hypothesis is put forward that the carbohydrates are absolutely essential for endo-cellular synthetic processes in connection with protein metabolism.

It is probable that food stuffs should be valued, as Chauveau suggested, more on account of their isoglycogenic than their isodynamic value.

The expenses of this investigation have been defrayed by a grant from the Carnegie Trust.

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ON THE REFRACTORY PERIOD OF MUSCLE AND NERVE.

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For every muscle or nerve which has hitherto been examined there is an interval immediately following excitation during which it is relatively difficult to produce excitation a second time. For convenience of reference we may provisionally call this interval, wherever it is observed, by the common name of refractory period, without any implied prejudice on the question whether the phenomenon is fundamentally the same in all tissues.

By investigators too numerous to be mentioned here the duration of the refractory period has been determined for a large number of different muscles and nerves, and shown to vary within very wide limits. It ranges from times of the order of ·001 sec. in the medullated nerve fibre of amphibia, to those of the order of 1 sec. in the cardiac muscle of amphibia and in the unstriated muscle of mammalia. But knowledge is altogether wanting on the fundamental question of the causation of the refractory period.

We are naturally inclined to regard the refractory period as the time of persistence of some process which must be brought to completion before it can be inaugurated a second time, but we gain little by this unless we are able to point to a definite one of the successive changes involved in excitation as the one actually responsible. There are certain well-known observations which seemed to me at first sight to afford a clue which might lead to the solution of this problem. Burdon-Sanderson[1] showed that the period of diminished excitability in the cardiac muscle of the frog corresponds approximately with the duration of the electric disturbance. Gotch[2] showed that in the

[1] This *Journal*, II. p. 427. 1879.

[2] *Ibid.* XXVIII. p. 48, Fig. 10. 1902.

medullated nerve of the frog a second stimulus produces no second response if it occurs before the electric disturbance produced by the first stimulus has passed its maximum and fallen again to about one-third of that value. The approximate coincidence of the refractory period with the duration of the electric disturbance in two tissues in which the refractory periods differ so widely suggests at once that the refractory period may be simply the time taken by the electric disturbance in subsiding to a certain fraction of its maximum value, a second excitation being impossible until the previous electric disturbance has been completed. In attempting to follow up this clue I proceeded first to enquire whether this coincidence could be verified in other muscles and nerves. The first which I chose for this purpose was the sartorius muscle of the frog. The results which I have obtained from the muscle fibres of this tissue have established one fact which puts the question in a new light. My experiments show that we have really two problems to face:—

(1) What determines the shortest possible interval between two effective stimuli.

(2) What determines the shortest possible interval between the beginning of one electric response and the beginning of a second electric response.

For the convenience of reference I shall proceed at once to distinguish these two intervals of time by different names. For the shortest interval between two effective stimuli I shall keep Marey's name, the Refractory Period. The shortest interval between two electric responses I shall call the *Irresponsive Period*. The necessity for this distinction arises from the fact, which is disclosed by my experiments, that in one and the same muscle under constant conditions the time interval between two successive stimuli may be varied within wide limits without any change in the time interval which separates the resulting electric responses. The second electric response appears at the termination of a definite irresponsive period whether the stimulus by which it is provoked occurs early or late in that period. The significance of this fact will be discussed more fully after the experimental evidence has been given.

Method. The experimental method consisted in recording with the capillary electrometer the electric response of the sartorius muscle of the frog in answer to two stimuli sent in at varying intervals of time. The stimulus used, the muscle chamber, the electrometer, the apparatus for recording and for analysis were in every detail those described in my

recent paper on the electric response of muscle[1]. For obtaining two stimuli at variable intervals of time two knock-down keys closing the primary circuits of two induction coils were arranged in the path of the lever which moves the photographic plate.

THE IRRESPONSIVE PERIOD.

I shall begin by describing the electric response of a sartorius, damaged by "thermal section" at the tibial end and led off from the middle and from the tibial end, to two stimuli sent in at the extreme pelvic end. For this purpose I reproduce in Figs. 1 to 6 a series of curves which are the results of analysis of photographic records made in the usual way. The abscissæ represent time, the ordinates P.D. between the electrodes.

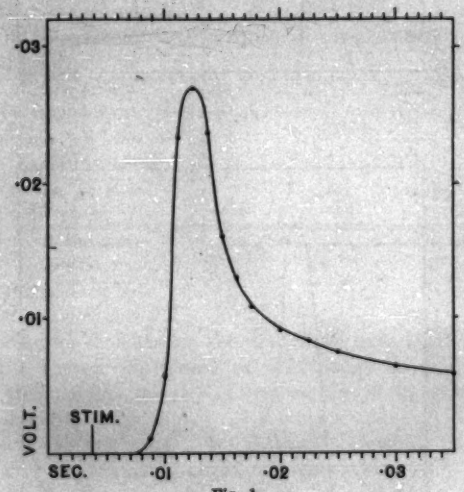

Fig. 1.

Fig. 1 is the response to the first stimulus sent in alone. Fig. 2 is the response to the stimulus which in the later observations was sent in at varying intervals after the first. It is important to notice that the response to the "second" stimulus is smaller than that to the first.

[1] This *Journal*, XXXIX. p. 207. 1909.

This is due to the second stimulus being less than the first. In all observations of this kind it is essential that the second stimulus should be made weaker than the first, because the experiment demands that the second stimulus should excite no fibres which have not been excited

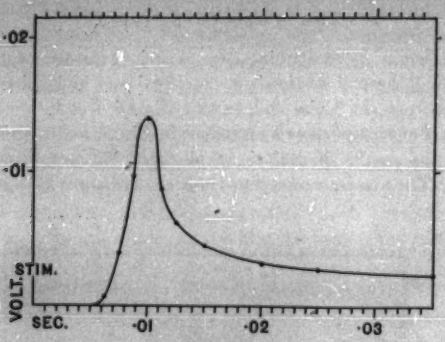

Fig. 2.

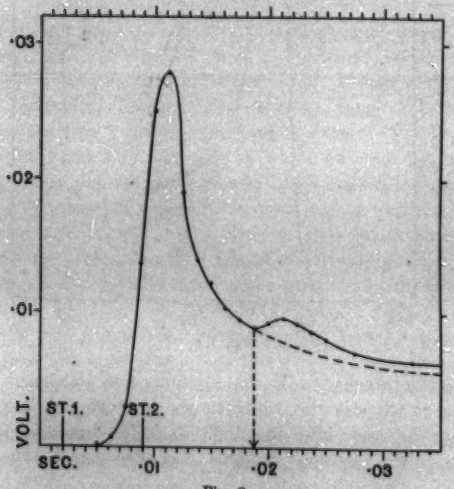

Fig. 3.

by the first. Figs. 3, 4, 5 and 6 are the responses to both stimuli sent in at intervals of ·0070 sec., ·0095 sec., ·0120 sec. and ·0172 sec. respectively.

On each of the curves 3 to 6 the broken line which continues the first response has been traced by superposition of the response to the single stimulus shown in Fig. 1. This broken line therefore represents the form which the curve would have had if the second stimulus had not been effective, and so enables us to determine with fair accuracy the point at which the second response first appeared[1]. From the point of appearance of the second response a vertical broken line has in each case been carried down on to the abscissa for the purpose of time measurement. If now we set out the time intervals between the first stimulus and the beginning of the response to the second we shall observe the remarkable fact that in the curves 3, 4 and 5, though the second stimulus occurs at widely different intervals after the first, the second response always begins at the same interval after the first stimulus, or in other words (since the electric responses to the first stimuli are practically identical) at the same interval after the beginning of the first electric response. The second response begins to appear later only in curve 6, where the second stimulus occurs so late that it is separated by a time less than the normal latent period from the point at which the earlier second responses appeared.

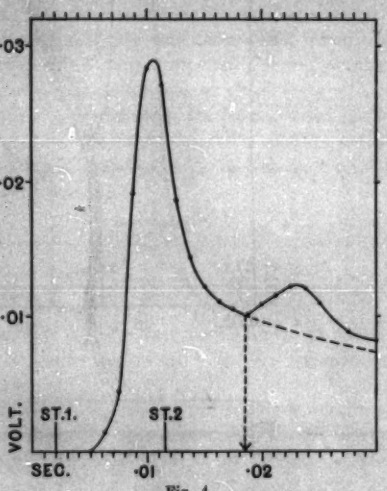

Fig. 4.

[1] The divergence between the responses to the first stimulus in Figs. 1, 3, 4, 5 and 6 is so slight as to be negligible. The curves are all plotted direct from the separate analyses of the several records.

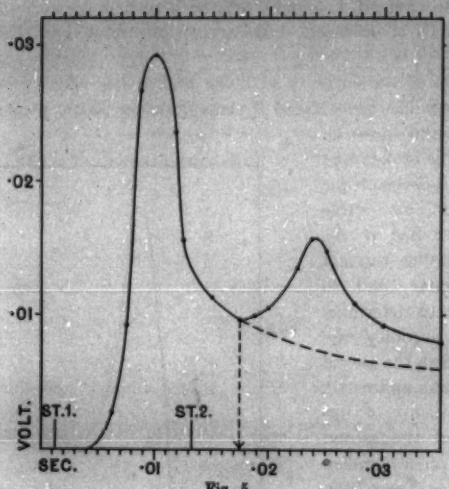
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Fig. 5.

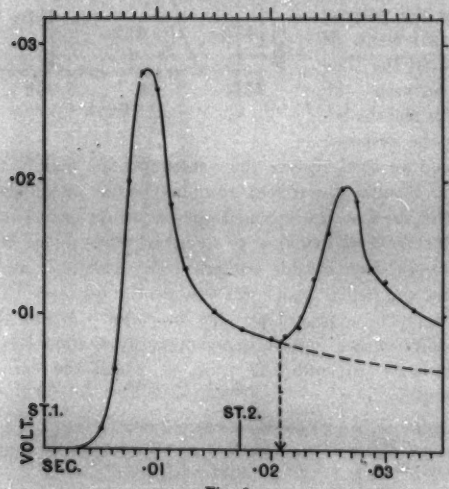
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Fig. 6.

Exp. 1. Sartorius of Frog at about 12° C.

Curve	Time interval from first stimulus to second	Time interval from first stimulus to beginning of second response
3	·0070 sec.	·0167 sec.
4	·0095	·0165
5	·0120	·0162
6	·0172	·0207

This experiment shows then there is a definite "Irresponsive Period" during which no second electric response can show itself. And the second response appears at the end of this irresponsive period whether the stimulus by which it has been provoked has occurred early or late during the period. If the stimulus occurs early, the latent period of the response is long; if it occurs late, the latent period is short. Actual measurements of the latent periods of the second response made on the curves 3 to 6 gave the following figures:—

Curve	Latent period of second response
3	·0097 sec.
4	·0070
5	·0042
6	·0035

In fact the latent period may be of almost three times its normal duration when the second stimulus occurs early in the irresponsive period.

In Fig. 7 there is reproduced an experiment which affords confirmation of the facts already described. In this experiment the tibial end of the muscle was not so completely injured as to produce a pure monophasic response; there was a slight electric disturbance under the second electrode.

In order to reduce the number of figures necessary, I have made a composite curve in which all the second responses are shown springing from the same first response. This is of course not strictly accurate since the first responses as measured from the separate photographic records did show a slight divergence among themselves. But the divergence consisted mainly of a variation of the maximum height of the curves within limits of about 7 %; in the part of the curve from which the second responses spring there was scarcely any perceptible difference[1]. The positions of the first stimulus (*A*) and of four second

[1] Greater accuracy of measurement is possible in this part of the curve, as the P.D. is measured almost entirely by the actual displacement of the meniscus, very little by its velocity. On the other hand the P.D. at the summit of the potential curve is measured mainly in terms of the angle of inclination of the photographic curve, and over a range of angles where the tangent changes rapidly with the inclination.

stimuli are shown. The response (diphasic) produced by the single stimulus *A* alone is the curve *AA*. Of the second stimuli *B* gave no second response (or in other words fell within the refractory period), *C*, *D*, and *E* gave responses (marked *C*, *D*, and *E*) which all began

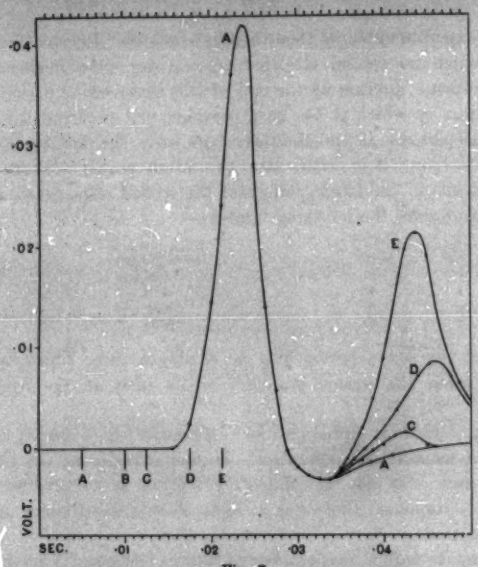

Fig. 7.

practically at the same point. A later second stimulus (*F*) which is not shown in the figure gave a second response which began a little later than the end of the irresponsive period. The actual measurements made on the original curves were the following:—

Exp. 2. Sartorius of Frog at about 12° C.

Curve	Time first stimulus to second	Time first stimulus to beginning of second response	Latent period of second response
B	·0050 sec.	no second response.	
C	·0075	·0295 sec.	·0220 sec.
D	·0125	·0295	·0170
E	·0162	·0292	·0130
F	·0249	·0320	·0077

Here we have again the same phenomenon, the second response occurring at the end of a definite irresponsive period whether the second stimulus was early or late, and occurring later only when the end of the irresponsive period was distant by less than the normal latent period from the second stimulus.

In the tables given above I have in each case shown the time interval between the first stimulus and the beginning of the second response, whereas our definition of the irresponsive period was the interval between the beginning of the first response and the beginning of the second. To arrive at the irresponsive period in each experiment we must therefore subtract the latent period of the first electric response from the interval which is tabulated. We may take the latent period (which of course is really in the main the time taken in conduction from the seat of excitation to the proximal leading-off electrode) as ·003 sec. in Exp. 1 and as ·0105 sec. in Exp. 2. These are the mean values obtained from measurement of all the curves as I drew them for the purpose of the other measurements given in this paper. The difference between the values obtained in the two experiments means merely that the proximal leading-off electrode differed in its distance from the seat of excitation. We have then for Exp. 1 the irresponsive period (·0165 sec.−·003 sec.)=·0135 sec., and for Exp. 2 (·0295 sec.−·0105 sec.) =·0190 sec., and so we see that in the sartorius muscle of the frog at about 12° C. a second electric response cannot follow a previous one at an interval less than about $\frac{1}{50}$th to $\frac{1}{100}$th of a second.

The relation between the refractory and irresponsive periods.

The importance of the experimental facts which I have described lies in the suggestion, to which they naturally point, that in looking for the cause of the refractory period we were dealing with a phenomenon of secondary importance, a mere corollary of the more fundamental irresponsive period. The discovery that the second electric response appears at a definite interval after the beginning of the first, without regard (within certain limits) to the time of occurrence of the second stimulus, is difficult to explain otherwise than on the view that some condition set up by the first excitation prevents directly and immediately the occurrence of a second electric response as such. And if this is so the refractory period may be merely a secondary result of the determinate time at which the electric response can appear; it may be for

example that the latent period of the electric response cannot have more than a certain duration, so that the refractory period is the irresponsive period plus the latent period of the first response minus the maximum latent period of the second response. Of course we have no proof that the refractory period is not a separate affair altogether, independently determined by some conditions other than the irresponsive period. But the direction of the enquiry must obviously be diverted to the irresponsive period which is shown to be of primary importance and away from the refractory period which may prove to be a mere corollary of the irresponsive period. Accordingly I am now engaged in ascertaining whether the irresponsive period can be seen in the cardiac as well as in the skeletal muscle of the frog, and, if so, whether it has in both tissues a constant relation to any recognisable process which results from excitation. Until these further data are available I postpone discussion of the conditions which determine the refractory and irresponsive periods.

Summary.

Two stimuli following one another at varying intervals are sent into the nerve-free part of the sartorius muscle, and the electric response of a neighbouring part of the muscle is recorded with the capillary electrometer. It is found that the second electric response begins at a constant time after the beginning of the first whether the stimulus by which it is provoked occurs immediately after the end of the refractory period or considerably later. If the second stimulus occurs immediately after the end of the refractory period the latency of the resulting electric response may be many times the normal. As the second stimulus is made later the latency of the resulting response becomes proportionately less. Only when the second stimulus occurs so late that it would otherwise have a latency less than the normal does the second response depart from its fixed time of occurrence.

The fact that the time of occurrence of the second electric response is constant, though the time of the second stimulus be varied, shows that the impossibility of obtaining a second electric response during an interval following a previous excitation is no mere secondary result of the existence of a refractory period (interval during which a second stimulus is not effective). To mark this distinction I propose the name "irresponsive period" for the interval between the beginning of one electric response and the earliest possible beginning of a second.

THE HEXONE BASES FROM EGG-WHITE.

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Historical. Siegfried[1] and Drechsel[2] were the first to separate hexone bases from the hydrolytic products of egg-white. Hedin[3] separated 2·4 gm. arginin from "300 gm. albumin aus Eiweiss (Grubler)" and later identified lysin[4] and histidin[5] among the products of dissociation of the same substance. Hugounenq and Galimard[6] found 2·15 % lysin, 2·14 % arginin and no histidin in the ovalbumin of hen's egg. Osborne, Jones and Leavenworth[7] found 4·91 % arginin, 1·71 % histidin, and 3·76 % lysin in crystallized ovalbumin from hen's eggs. Osborne, Leavenworth, and Brautlecht[8] mention also the figures for arginin, histidin and lysin from conalbumin from hen's eggs.

Scope of investigation. The amounts of arginin, histidin and lysin have been estimated in the products of the hydrolysis of egg-white. The whole egg-white rather than any separated protein has been employed since egg-white is used for experimental work in nutrition.

In certain preliminary attempts the hydrolysis has been performed with 5 % sulphuric acid and also (by one of us, H.G.C.) with pancreatic juice obtained by the injection of secretin into a dog and activated by enterokinase. These hydrolyses were incomplete though continued for many hours. Complete hydrolysis has been accomplished with 25 % sulphuric acid.

[1] *Ber.* XXIV. S. 418. 1891.
[2] *Arch. Anat. u. Physiol.* S. 271. 1891.
[3] *Zeit. physiol. Chem.* XXI. S. 163. 1895—6.
[4] *Ibid.* XXI. S. 302. 1895—6.
[5] *Ibid.* XXII. S. 191. 1896—7.
[6] *C. R.* t. 143, p. 242. 1906.
[7] *Amer. Journ. of Physiol.* XXIV. p. 252. 1909.
[8] *Amer. Journ. of Physiol.* XXIII. p. 194. 1908.

Methods. The methods of separation devised by Kossel and Kutscher[1] with the modifications of Kossel and Patten[2] and Osborne, Leavenworth, and Brautlecht[3] have been employed. In four out of five determinations the hexone bases were first precipitated by phosphotungstic acid. In this way interference by reducing bodies with the precipitations by silver salts was avoided.

Hydrolysis. A typical experiment was as follows:—680 c.c. egg-white containing 95·7 gm. solid matter, 89·6 gm. protein, and 12·32 gm. nitrogen were placed in a steam sterilizer with 50 c.c. sulphuric acid previously mixed with 150 c.c. water for seven hours.

Then 200 c.c. sulphuric acid were added and the mixture steamed for six hours. It was then boiled for 69 hours on a sand-bath with a reflux condenser. The volume was made up to 1 litre and 10 c.c. removed for a determination of amide ammonia and melanoidin[4]. Previously attempts had been made to perform the hydrolysis with 5 % sulphuric acid. Thus 39·25 gm. dried egg-white were boiled with 500 c.c. 5 % sulphuric acid for fifty hours. After twenty-five hours the biuret test was positive, but at the end it appeared to be negative. Two analogous experiments were carried out. In these and also in the tryptic digest the amounts of hexone bases were too small and the quantity of nitrogen precipitated by phosphotungstic acid too large. Certain of the final products were contaminated with bodies resembling the dipeptide isolated by Levene and Beatty[5]. Corroboration was obtained of the difficulty in completing the hydrolysis of some proteins as noted by Osborne, Leavenworth, and Brautlecht.

Separation of the hexone bases by phosphotungstic acid. Filtered digests were diluted to contain 5 % sulphuric acid. Phosphotungstic acid was added until the five litres contained 5 % reagent. Further precipitation that occurred on the addition of more phosphotungstic acid consisted only of reagent. After seventy-two hours, the superfluid was separated in the centrifuge. All separations and washings of precipitates have been done in the centrifuge. Much labour and time is saved in this way. The various stages in the separation were controlled from this time onwards by nitrogen determinations. In calculating the figures no allowance has been made for the loss in the large precipitates.

[1] *Zeit. physiol. Chem.* XXXI. S. 165. 1901.

[2] *Zeit. physiol. Chem.* XXXVIII. S. 39. 1903. [3] *Loc. cit.*

[4] Estimated after the method of Osborne and Harris, *Journ. Amer. Chem. Soc.* XXV. p. 346. 1903.

[5] *Biochem. Zeit.* IV. S. 299. 1907.

Separation of histidin from arginin. The separation of arginin and histidin was in our hands difficult.

It was invariably found that arginin was precipitated with histidin in the second silver precipitation of Kossel and Kutscher. This was obvious upon applying Kossel and Patten's process to the histidin portions. Nitrogen determinations showed the presence of arginin in the filtrate from mercuric sulphate. Further, it seemed that mercuric sulphate did not rapidly precipitate histidin, as four or five precipitates containing histidin separated from the filtrate if this were allowed to stand three or four weeks.

On employing precipitation by mercuric sulphate previous to the second silver precipitation as suggested by Osborne, Leavenworth, and Brautlecht no difficulty was experienced in the separation.

Estimation of the separated hexone bases. Two methods have been previously employed. In the first method the amount of nitrogen is estimated in the final solution and the quantity of base calculated from this figure. The bases are isolated only for identification. In the second method the isolated bases are used to calculate the weight of base.

While little can be said against the practice of calculating the weight of base present in the "purified" fluids from which the salts are crystallized, there is more doubt concerning another practice recently introduced. Jackson and Pearce[1] estimated the total hexone bases from the amount of nitrogen precipitated by phosphotungstic acid, the arginin and histidin from the nitrogen precipitated by silver nitrate and baryta, and the lysin from the nitrogen in the filtrate freed from silver and baryta. Siegfried and Pilz[2] adopted a somewhat similar practice. The results recorded from our experiments in Table I. show that both these portions (histidin plus arginin, and lysin) contain nitrogen not attributable to these substances.

Results. A record of two experiments is given in Table I.

The amounts of nitrogen obtained at the various stages of the separation are stated. The first hydrolysis was performed with 5 % sulphuric acid for seventeen hours, the second with 25 % sulphuric acid for sixty-nine hours. The first separation was according to Kossel and Patten, the second by the modification of Osborne, Leavenworth, and Brautlecht. In each case the precipitation with silver and baryta was repeated to throw down completely arginin.

[1] *Journ. of Expt. Med.* IX. p. 520. 1907.
[2] *Zeit. physiol. Chem.* LVIII. S. 224. 1908.

The percentage of nitrogen present in each stage enables the two results to be compared. The amide N is 8·52 % compared with Hausmann's 8·53 %, while the melanoidin N is 1·82 %.

The high figure for the precipitate with phosphotungstic acid in the first hydrolysis (30·5 %) is due to the precipitation of polypeptides. This was evidenced by the separation with the lysin of the body resembling the dipeptide of Levene and Beatty[1].

TABLE I.

	Hydrolysis I		Hydrolysis II	
	Gms. N	Per cent. of total N	Gms. N	Per cent. of total N
N in hydrolysed fluid	2·15	100·0	12·32	100·0
N as ammonia (amide N)	—	—	1·05	8·52
N as melanoidin	—	—	·224	1·82
N pptd. by phosphotungstic acid after removal of ammonia	0·656	30·50	2·140	17·36
N pptd. by silver and baryta	·238	11·07	1·045	8·48
N in filtrate from silver and baryta (by difference)	·418	19·43	1·095	8·88
N in filtrate from silver and baryta pptd. by phosphotungstic acid	·147	6·84	0·548	4·45
this gives N as lysin	(·054*	2·51*)		
N in histidin portion	·0293	1·37	0·160	1·30
N in arginin portion	·117	5·44	·685	5·56
N pptd. by silver and baryta and not pptd. as histidin or arginin	·038†	1·77	·200†	1·62
N in filtrate from silver and baryta and not pptd. by phosphotungstic acid ...	—	—	·190†	1·55

* Calculated from the separated lysin picrate.

† Amount actually estimated, the remainder unaccounted for.

Owing to this admixture the lysin nitrogen was calculated from the lysin picrate as 0·054 gm. and not from the nitrogen precipitated by phosphotungstic acid, viz. 0·147 gm. On the other hand 17·36 % in the second hydrolysis is a low figure since the method of Hausmann[2] gives 21·33 %. Our low figure is due to incomplete washing of the precipitate of barium sulphate and phosphotungstate. It must be pointed out that loss of nitrogen occurred with each precipitate of barium sulphate so that the nitrogen figures do not add up. Thus in Expt. 2, 0·548 gm. N as lysin plus 0·19 gm. N other than lysin or 0·74 gm. N were obtained from 1·095 gm. N present originally.

The figures for histidin and arginin agree well.

[1] *Loc. cit.*

[2] *Zeit. physiol. Chem.* XXVII. S. 95. 1899.

The protein in the second hydrolysis amounted to 89·6 gm. The lysin calculated from the nitrogen present in the solution from which the lysin picrate was precipitated was 2·86 gm. The lysin picrate once crystallized weighed 5·47 gm. 0·133 gm. lysin picrate gave 17·5 c.c. $\frac{N}{10}$ NH_3 by Kjeldahl-Jodlbauer-Gunning method.

$C_6H_{14}N_2O_2.C_6H_2(NO_2)_3OH$ requires 18·66 % N. Found 18·42 % N.

The solution of histidin contained nitrogen equal to 0·59 gm. histidin. There was crystallized 0·25 gm. histidin dichloride and 0·484 gm. histidin picrolonate.

0·0618 gm. histidin dichloride gave 0·0762 gm. AgCl.

$C_6H_9N_3O_2.2HCl$ requires 31·18 % Cl. Found 30·5 % Cl. The histidin dichloride gave a slight residue of barium salt on combustion.

The solution of arginin contained nitrogen equal to 2·14 gm. arginin. A portion was lost, but from a fraction containing 0·433 gm. nitrogen 2·437 gm. arginin nitrate were obtained.

The arginin nitrate was converted into arginin copper nitrate.

0·219 gm. arginin copper nitrate gave 0·020 gm. H_2O and 0·0237 gm. Cu.

$(C_6H_{14}N_4O_2)_2.Cu(NO_3)_2.3H_2O$ requires 10·79 % Cu and 9·16 % H_2O. Found 10·83 % Cu and 9·14 % H_2O.

The amounts of arginin, histidin, and lysin present in 100 gm. protein of egg-white are therefore:

Arginin	2·39 %,
Histidin	0·66 %,
Lysin	3·19 %.

In conclusion we express our thanks to Professor Anderson Stuart in whose laboratory this investigation was conducted.

NOTE ON ASPHYXIA IN THE SPINAL ANIMAL.

By R. KAYA (of Kioto) and E. H. STARLING.

(*From the Institute of Physiology, University College.*)

Among the various phenomena which attend the production of asphyxia in the warm-blooded animal, the most prominent are those affecting the skeletal muscles and the circulatory system. In the production of these phenomena the essential factor is a change in the gaseous contents of the blood, which, in asphyxia as ordinarily induced, affects both the carbon dioxide and the oxygen content of this fluid. In a series of experiments carried out by Dr Scott in this laboratory, we had occasion to observe the relative part played in the production of the muscular phenomena of asphyxia by the two factors whose action is generally combined, namely excess of CO_2 and diminution of oxygen. As is well known, if an animal be allowed to breathe air containing an excess of carbon dioxide (from 2 to 10 %), a condition of dyspnœa is produced, which, as Haldane has shown, is sufficient, with percentages of CO_2 in the air up to 6 %, to maintain the alveolar tension of this gas approximately constant. In this dyspnœa the displacement of oxygen in the air by the CO_2 added is not involved, and the phenomena are the same if, by the artificial addition of pure oxygen to the mixture breathed, its content in oxygen is brought up to between 25 and 40 %. In this increased activity, the respiratory movements are perfectly coordinated. Convulsions are not produced by carbonic acid, though there may be some forced reflex movements with higher percentages of this gas in consequence of direct stimulation of the tracheal mucous membrane. Continued inhalation of high percentages of CO_2 with oxygen tends finally to bring about a condition of narcosis and to lower thereby the activity of the respiratory centre.

When we study the influence of diminution of oxygen in the blood without any accumulation of carbon dioxide, as for instance, by making the animal breathe pure nitrogen or nitrogen mixed with a small

percentage of oxygen, the phenomena observed present several points of difference from those just described. A diminution of the oxygen-content in a mixture of oxygen and nitrogen from 20 to 14 %, has, as a rule, in the animals which we have observed (generally chloralised rabbits) no effect at all on the rhythm or depth of respirations. On still further diminishing the percentage of oxygen to 8 or 10 %, an influence on the respiratory movements is observed almost identical with that produced by the inhalation of low percentages (3 and 4 %) of CO_2. There is in the animal with intact vagi increased amplitude and rhythm of the respiratory movements. This condition of hyperpnœa may last for some minutes. On still further reducing the oxygen to a point which varies with the depth of anæsthesia of the animal, but which may as a rule be put between 4 and 8 %, there is at first a condition of hyperpnœa in which the respiratory movements are simply quickened and deepened but remain perfectly coordinate. After these movements have lasted for 20 to 30 seconds however, there is a sudden convulsive spasm apparently affecting all the muscles of the body, but resulting mainly in an expiratory effect. This spasm may be repeated at shortening intervals, becoming more extensor or inspiratory in character until the death of the animal occurs. On the other hand it is often possible to find a strength of the gaseous mixture in oxygen which produces first hyperpnœa, then one or more convulsions, which however do not recur, so that the animal goes on breathing regularly as long as the gaseous mixture is administered. We have observed this for instance with as low a percentage of oxygen as 5 %, and have ascribed it to a lowering of the excitability of the nervous centres as a result of the partial oxygen starvation. If we are recording the respiratory movements by Head's method, it may be observed that the asphyxial convulsion does not replace the rhythmic movements of the diaphragm slip, but is, so to speak, superadded to it, so that although the tracing is more or less deformed by the alteration of the fixed point from which the muscular slip is acting, brought about by the convulsive muscular contractions, the contractions of the slip continue at the same rhythm as before. The impression one obtains from such experiments is that two mechanisms are really involved in the motor phenomena of asphyxia, namely, that the respiratory centre is stimulated to increased coordinate efforts to ærate the blood properly, but when the oxygen content of the blood falls sufficiently low, some other lower centres are excited with the production of general muscular spasm, which breaks in upon and confuses the rhythmic respiratory movements presided

over and effected by the respiratory centre. Since this interruption of the increased respiratory activity is not brought about under the influence of increased CO_2 tension in the blood, it seemed to us that this lower centre would be stimulated only by oxygen lack. An investigation of the reaction of the spinal cord to asphyxia ought therefore to show that while this, like the rest of the central nervous system, presented a primitive function of excitation by lack of oxygen, it would not partake of the sensitiveness to increase of CO_2 tension, which has been evolved in the medullary centres of the air-breathing animals.

The excitation of the medullary centres by asphyxial conditions of the blood, is not confined to the respiratory centre. We may indeed speak of a general medullary discharge which may affect blood-vessels, heart, glands, etc. Whether this general excitation of medullary centres is primarily aroused in the respiratory centre, it is impossible to say, but since the vascular phenomena, especially vaso-constriction, are observed in the spinal animal, as in the animal with intact medulla, it is important to differentiate also between the influence of increased CO_2 and lack of oxygen respectively in the production of vascular constriction and rise of blood pressure by asphyxia. It is a familiar fact that the result of asphyxia on the blood pressure is very variable. In some cases there is a very large rise of blood pressure: in other cases the rise is small or absent, the cessation of the æration of blood being rapidly followed by heart failure and a fall of pressure. The factors involved in these changes are seen clearly if we study separately the effects of increased CO_2 and of diminished oxygen on the blood pressure. Since the increased movements produced by these two conditions might bring about mechanical changes in the blood pressure, it may be said at once that the changes observed are practically identical, whether the muscular movements be prevented by previous injection of curare or not. If the animal be made to breathe a mixture containing about 5 % CO_2 with excess of oxygen, the increased activity of the respiratory centre is accompanied by increased activity of the vaso-motor centre. There is a steady rise of blood pressure during the inhalation which goes on until the blood pressure attains a height which may be double that ruling at the beginning of the experiment. If the inhalation be continued the blood pressure remains at this height and no sign is seen of any heart failure, in fact, the heart beats are more powerful than they were at the beginning of the experiment. On ceasing the inhalation of CO_2 the blood pressure falls slowly to its previous height and attains this height

in the non-curarised animal at about the same time that the respiratory movements have returned to normal. If the animal (dog, cat or rabbit) be made to inhale pure nitrogen or nitrogen containing 5 % or less of

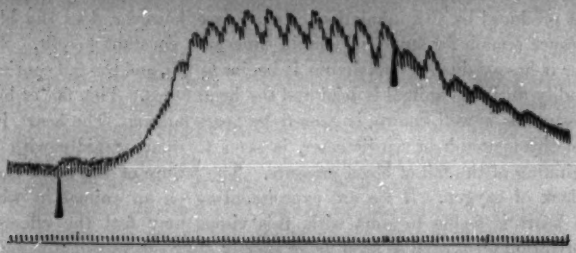

Fig. 1.

Blood pressure curve from curarised dog (morphia and urethane anæsthesia). Both vagi cut. Between the two arrows, a gaseous mixture containing 7 % CO_2 and about 25 % oxygen was used for artificial respiration. Time marking = 2 secs. Zero-pressure corresponds to limit of time marking.

oxygen, the effect observed in most cases is at first similar to that produced by excess of CO_2. There is a steady rise of pressure accompanying the hyperpnœa produced under these circumstances. Suddenly, however, the rate of rise of the arterial pressure is increased, and almost immediately, within two or three seconds, there is a general convulsion, during which a rapid and extensive rise of blood pressure

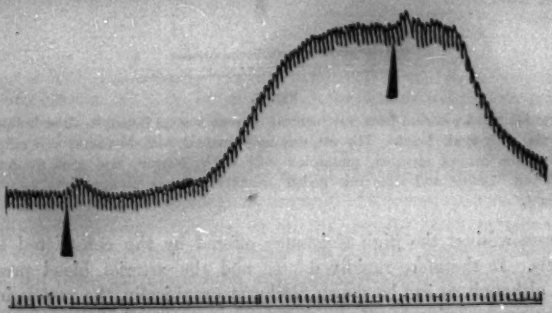

Fig. 2.

Blood pressure curve from dog (morphia urethane and curare). Between the arrows almost pure nitrogen (containing 0·5 % oxygen) was administered.

occurs. With the cessation of the convulsion the blood pressure falls to a certain extent, to rise again with the renewed convulsions. In curarised animals the same slow rise followed by a rapid rise may be observed in the absence of convulsions and the curve is very similar to that produced by CO_2 inhalation. In no case, however, does the blood pressure remain for any considerable time at a constant height. Soon after it has attained its maximum, it begins to fall, and the fall continues steadily until the animal is dead and the heart stops. This fall of blood pressure, as is well known, is caused by heart failure. The heart, if in a plethysmograph or cardiometer, is seen to dilate rapidly with the beginning of the fall of blood pressure. The failure of the heart is due to lack of oxygen. If we are experimenting on an animal in whom the heart is feeble to start with, this viscus may feel the effects of deprivation of oxygen as soon as or shortly after the vaso-motor centre or centres have been stimulated. As a result, the heart is unable to carry out any longer its normal amount of work, much less then to

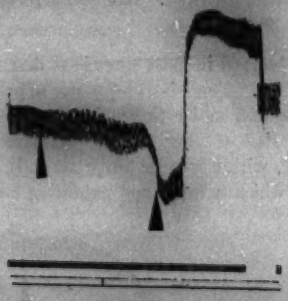

Fig. 3.

Arterial blood pressure from cat (central nervous system intact) to show influence of asphyxia with weak heart. The cat was anæsthetised with urethane, and curarised. Between the arrows nitrogen, containing only 0·5 % oxygen, was used for artificial respiration. Before and after this period ordinary air was pumped into and out of the chest.

contract against the high resistance offered by the constricted blood vessels. It therefore rapidly dilates and the arterial blood pressure falls, although, as can be shown plethysmographically, the vessels in the organs are constricted. Under these circumstances, it is often possible, without the use of the plethysmograph method, to demonstrate the existence of general vaso-constriction. If when the blood pressure is

falling, the administration of oxygen-free air be stopped and artificial respiration with oxygen or ordinary air be set up, the oxygenised blood travels from the lungs into the heart, and gets into the heart muscle along the coronary vessels several seconds earlier than, in the onfeebled condition of the circulation, it can arrive at the central nervous system. By this access of oxygen the heart muscle acquires power to contract even against the increased resistance offered by the still constricted arterioles, and there is therefore a rapid rise of blood pressure which may far exceed the primary rise observed on the induction of asphyxia, and may be pronounced when the rapid heart failure prevented any asphyxial rise of blood pressure being produced.

From Sherrington's observations we know that asphyxia of the spinal animal evokes not only spasm of the skeletal muscles, but also spasm of the blood vessels with consequent large rise of blood pressure. So far as we are aware, no attempt has yet been made to dissociate the relative parts played in this condition brought about by oxygen-lack and CO_2-excess respectively. An investigation of this question shows conclusively that there is a marked difference between the behaviour of the spinal cord and the spinal cord *plus* medulla with respect to these

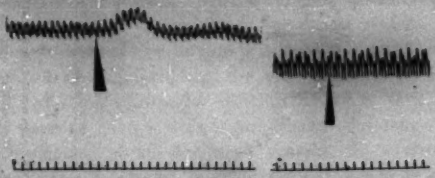

Fig. 4.

Arterial blood pressure tracing from decapitated cat. Between the arrows a mixture containing nitrogen, 13·7 % CO_2 and 21·2 % oxygen was administered. The interruption in the tracing corresponds to a period of 2 mins. 44 seconds. The general effect was to cause a slight fall of blood pressure.

two gases. Working with a spinal animal, the administration of carbon dioxide in the presence of adequate amounts of oxygen produces no result, either on the skeletal muscles or on the general blood pressure. In some cases we have noted an increase in the amplitude of the heart-beats, but there has never been any appreciable rise of blood pressure. On the other hand, if the air used in artificial respiration be replaced with pure nitrogen, or with nitrogen containing 5 % or less of oxygen, there is invariably, some time after the beginning of the asphyxia, a general spasm of all the muscles of the body, accompanied or slightly preceded by a marked rise of arterial blood pressure. With regard to

the latter phenomenon we may observe similar individual variations to those which may be obtained in the intact animal. If the heart is in a feeble condition at the outset, oxygen want may bring about a primary fall of blood pressure, but the presence of vascular constriction is shown

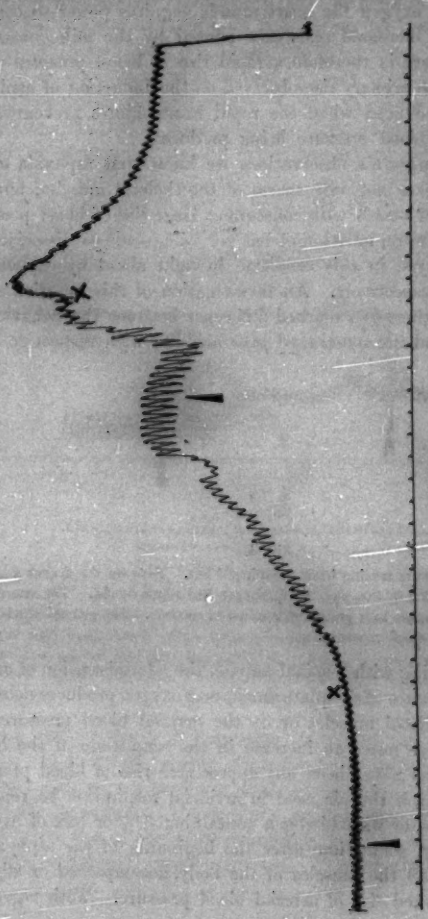

Fig. 6.

Arterial blood pressure tracing from decapitated cat. Between the arrows nitrogen (with 0·5 % oxygen) was administered. A general muscular spasm occurred at the first cross. The sudden rise at the second cross was unattended by any muscular spasm. Time marking = 6 seconds.

at once on renewing respiration with air or oxygen, when a marked rise of blood pressure is immediately produced. These facts are well illustrated in the accompanying curves reproduced from the tracings of arterial blood pressure in spinal animals.

Conclusions.

In the ordinary phenomena of asphyxia, two sets of phenomena are involved.

(1) The increased tension of CO_2 in the blood or in the nervous tissues. This acts entirely on the centres in the medulla oblongata and possibly on those in the higher parts of the brain. As a result, there is increased activity of all these centres with the production of hyperpnœa or dyspnœa, i.e. exaggerated but coordinated respiratory movements and a steady rise of arterial blood pressure. A moderate increase of the carbon dioxide tension in the blood has no injurious effect on the heart, and may possibly in fact improve the functional capacity of the heart muscle.

(2) Diminished oxygen content in the blood with consequent lack of oxygen supply to the tissues. This factor acts probably on the whole central nervous system, but certainly on the spinal centres. When the oxygen tension is lowered to a certain point, there is a sudden excitation of all the spinal centres, including the motor centres for the skeletal muscles and the local vaso-motor centre. There is thus a universal discharge of nervous energy with a production of spasm of the whole body and constriction of all the blood vessels. In curarised animals the muscular spasm is absent, but the arterial spasm is produced as before with, as a result, a rise of blood pressure. This excitatory effect is followed by marked depression of all the spinal centres. On the heart the effect of oxygen lack is primarily depressor. No stage of increased activity is to be observed, and the depression of the heart's activity may be so marked that it may counteract the influence of the constriction of the blood vessels on the arterial blood pressure. We thus get a primary fall instead of rise of arterial blood pressure.

We may thus speak of the phenomena of asphyxia as primarily determined by the excitation of the medullary centres by the increased CO_2 tension. If, however, this increased CO_2 tension is accompanied, as is usually the case, by a diminished oxygen tension, the lower centres are excited and the effects of their excitation break in upon and disorder the phenomena due to the increased functional activity of the medullary centres.

THE ACTIVE PRINCIPLE OF A BINI SPEAR POISON.

By P. P. LAIDLAW.

(From The Wellcome Physiological Research Laboratories, Herne Hill, S.E.)

THROUGH the kindness of S. Sproston, Esq., I have been able to examine two spear heads poisoned for elephant hunting, which were obtained from West Africa. They were obtained by him from a Bini huntsman of Benin City, who is the only successful maker of the poison in the district. The composition and method of preparation of this poison is a secret handed down from father to son. The huntsman would not disclose the actual ingredients of the poison, but stated that vegetable matter only was used in its manufacture, that it took two or three months to prepare, and that it retained its activity for years. The use of poisoned spears and arrows in elephant hunting has been recorded before, but owing to the large size of the animal and the small dose of the poison introduced by a spear or arrow wound there is usually a long time interval ("half a day") between the wound and the death of the quarry.

The Niger District is quoted by Fraser[1] among eight others as a locality in which the natives use strophanthus as an arrow poison. The Munchi[2] arrow poison also comes from this district.

The two iron spear heads were thickly coated all over with a dark brownish green coloured mass of glue-like consistency. An extract in ·6% salt solution was made and injected into an intact frog. At the end of half an hour no sign of heart beat could be detected, and reflexes were very feeble. In a few minutes more no response could be elicited to mechanical stimulation. Post-mortem the ventricle was pale and firmly contracted, the auricles engorged with blood; central and peripheral electrical stimulation of the cut sciatic nerve evoked no response

[1] Fraser. *Trans. Roy. Soc. Edin.* XXXV. p. 955. 1889.

[2] Cf. Fröhlich. This *Journal*, XXXII. p. 319. 1905. Also Mines. *Ibid.* XXXVII. p. 37. 1908.

in the muscles though they responded well to direct stimulation. A 1% extract of the poison in ·6% saline was found to be very active. ·05 c.c. of such an extract killed a 30 gram frog in the course of two hours. The ventricle was arrested in systole. These experiments were

Fig. 1. Appearance of spear heads
A. Before removal of poison,
B. After removal of poison.
Greatest length 5¼ inches, greatest breadth 1½ inches.

repeated with similar results—systolic arrest of the ventricle, in some instances a curare-like effect on motor terminations, and a feeble or absent response to central stimulation of the cut sciatic.

A solution of the poison in saline lost activity on hydrolysis with hydrochloric acid. ½ c.c. and 1 c.c. of a dilute solution of the poison proved fatal to two frogs in an hour and a half. This solution was hydrolysed with hydrochloric acid, neutralised, brought back to original volume. ½ c.c., 1 c.c. and 1½ c.c. injected into frogs had no perceptible effect, and two days afterwards the frogs were alive and appeared normal.

A poison with such high toxicity and characteristic physiological action, is probably strophanthin. Recognised methods for the isolation of strophanthin were therefore employed. The poison was scraped off the spear heads after a preliminary soaking in 70% alcohol. It was then

ground up in a mortar with a little alcohol and extracted twice with warm 70 % alcohol. The solid left behind was only slightly active. It contained some hard gritty particles, but for the most part consisted of disintegrated vegetable matter (microscope).

The alcoholic extract was very active. It was evaporated down to small bulk when some dark brown oily drops separated. Some water was added and the whole precipitated with basic lead acetate. The lead precipitate carried down all the finely divided oil drops and left a clear yellow solution. The excess of lead was removed by sulphuretted hydrogen, the filtrate taken to a small bulk and set aside in a desiccator in the hope that the strophanthin would crystallise out (Feist[1], Kohn and Kulisch[2]). The solution became a thick gum. This being unsuccessful the gummy mass was dissolved in water and saturated with ammonium sulphate (Thoms[3]): a slimy precipitate separated which was taken up by amyl alcohol. The residue after evaporating off the amyl alcohol was taken up in alcohol and attempts at crystallisation were made from water, alcohol, methyl alcohol, acetone, all of which freely dissolved the product, but all attempts failed. The product was insoluble in chloroform, ether, acetic ether, and petroleum ether. Precipitation with these did not appear to purify the product further. It has a bitter taste, and when finely powdered has an irritating effect on the mucous membrane of the nose. It gives a fine green colour with strong sulphuric acid which becomes a brown green on standing.

On hydrolysis it yields a reducing sugar from which however no crystalline osazone was obtained (see Feist, *loc. cit.*).

The product was tested on frogs, cats and rabbits. In all cases the mode of death was by arrest of the heart (ventricle in systole). The minimum lethal dose was determined for frogs using a three hour limit: 0·12 mgrm. per 100 grms. killed within the limit and 0·1 mgm. per 100 outside the limit. The minimum lethal dose of strophanthin from strophanthus gratus (the most potent of the strophanthins) per 100 grm. frog is ·07—·08 mgrms[4].

Three or four mgrms. of the product, when injected into a cat produced a rise of blood-pressure and some slowing of the heart beat, a little later the heart became very rapid and irregular, the blood-pressure rose still further and showed undulations; ultimately it

[1] Feist. *Chem. Centralb.* I. p. 946. 1898.
[2] Kohn and Kulisch. *Ibid.*
[3] Thoms. *Ber. d. Deut. pharmaceut. Gesellsch.* p. 104. 1904.
[4] Ziegenbein. *Ber. d. pharm. Gesellsch.* XII. p. 335. 1902.

somewhat suddenly fell to zero. Post-mortem the ventricles were found firmly contracted.

Isolated rabbits' hearts perfused with Ringer's solution using the Locke technique, showed augmentation of beat when the drug was perfused through the heart in a concentration of 1 in 200,000. A little later delirious irregularity set in and the heart stopped in systole. Fig. 2 shows the several stages in such an experiment.

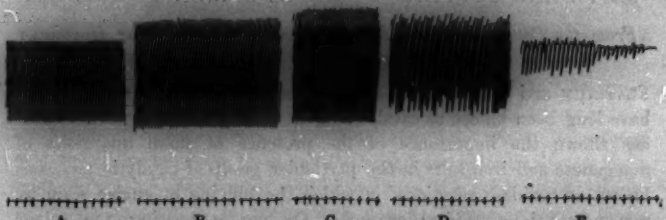

Fig. 2. Isolated rabbit's heart, Locke-Langendorf method.

A. Normal.
B. After 50″ perfusion with 1 in 200,000 crude strophanthin from spear poison.
C. 2 minutes later.
D. 70″ later.
E. 70″ later.
Upstroke = systole, time = seconds.

It seems clear that the active principle of the poison belongs to the class of glucosides, gives the characteristic colour reaction of Kombé strophanthin, and possesses the solubilities and physiological properties of that substance.

The rather high minimum lethal dose for the purest product obtained, is quite consistent with this view since certain impurities must have been present which prevented crystallisation. Two white substances were isolated from the poison, which crystallised in needles. As they were inactive when tested physiologically they were discarded.

Attempts to make crystalline strophanthidin from the remainder of the product failed.

THE CATALYTIC OXIDATION OF GUAIAC RESIN BY METALLIC COPPER.

By HECTOR A. COLWELL, M.B.

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Platinum and certain other metals, when in a fine state of division, have long been known as catalytic agents. More recently, Bertrand[1] has shown the importance of the presence of small quantities of manganese and iron salts in the particular group of catalytic processes known as 'oxidase reactions,' of which the blueing of guaiac resin by raw potato is a typical example. On gentle heating the colour develops more rapidly, and, if the heating be continued, it disappears, to reappear on cooling. If the heating be excessive, the blue colour does not reappear on cooling, owing to the destruction of the enzyme which produced it.

In the foregoing instances, any metal present is either in a finely divided form (platinum or palladium black) or is present as a salt (*e.g.* oxidases). The reaction under consideration differs from both these, inasmuch as it is brought about by ordinary metallic copper, preferably in the form of gauze. Other metals (*viz.* silver, mercury, lead, tin, aluminium, zinc, iron, gold and platinum) were tried, under similar conditions, but did not show this property.

If a strip of copper gauze be placed in a solution of guaiac in 90% alcohol at the room temperature, the solution gradually acquires a deep blue colour. A like blueing occurs, though more slowly, on introducing a similar strip into an emulsion made by adding guaiac tincture to distilled water. The phenomenon is identical with that produced by potato oxidase.

(1) *Effect of heat and cooling.* In all three cases the reaction is accelerated by warming, and the colour makes its appearance gradually. If the blue liquid obtained by the action of metallic copper upon guaiac solution be evaporated to dryness the colour disappears, and the residue has all the appearances of ordinary guaiac resin. It can, moreover, be dissolved in alcohol as before, and behaves as unaltered guaiac, both to

[1] Bertrand, *Compt. rend.* 1897, T. 124, p. 1032.

copper and to oxidizing enzymes. In the case of the emulsion blued by copper, if the metal be removed, and the liquid briskly boiled, the colour goes in the same way as when guaiac emulsion blued by potato oxidase is heated, and similarly does not reappear on cooling; if, however, the copper be allowed to remain throughout, the colour reappears as the mixture cools. If the watery emulsion, previously blued by copper, be shaken with chloroform and allowed to stand, the chloroform extract sinks to the bottom of the tube as a blue liquid, leaving the supernatant fluid comparatively clear and colourless. On separating, and evaporating the extract to dryness the residue again has the appearance and reactions of ordinary guaiac.

(2) *The effect of reducing agents.* If a solution of H_2S in water be added to the blue alcoholic solution, first described, an immediate discharge of colour occurs. The blue emulsions which have been coloured either by copper or by potato are also decolourized though much more slowly. A zinc-copper couple produces the same effect, though the action is again in all cases much slower. Reducing agents therefore act on the substance produced by copper in the same way as they act upon that produced by oxidases.

(3) *The presence of oxygen.* The presence of oxygen has previously been stated to be necessary for the action of oxidases, and to determine its relation to the reaction produced by copper the following experiment was performed. Into two tubes were introduced 1 c.c. of a strong alcoholic solution of guaiac; 15 c.c. of ordinary distilled water were added to the first tube. To the second tube was added an equal bulk of water which had been well boiled for an hour, and allowed to cool. Both tubes were corked, and the first (control) tube thoroughly shaken to ensure solution of as much air as possible. Equal sized strips of copper gauze were introduced into both tubes, the corks replaced, and the whole put into an incubator kept at 55° C. The progress of the reaction was observed through the glass door of the incubator. The control tube, in which the ordinary water had been placed, acquired a blue tint much more rapidly than the other, though after an hour or thereabouts the colours were identical. The presence of a sufficiency of oxygen therefore appears to be as necessary in this case as in that of oxidase.

(4) *The presence of water.* Guaiac resin was dissolved in the following reagents, and to a sample of each solution a strip of copper gauze was added,—methyl alcohol, amyl alcohol, acetone, chloroform, toluol, xylol, ethyl bromide, ether and benzol. The result was the same as when the solution in 90 % alcohol was used, except in the case

of ether and benzol. Now all the other solvent media employed were ordinary laboratory reagents in more or less constant use, and consequently contained fair traces of water absorbed from the air. The benzol and ether, on the other hand, were specially pure samples in bottles not previously opened, the reactions obtained with solutions in these liquids were of excessively slow development: the benzol solution was left in contact with copper for four hours at 55° C. with only a very small change (under ordinary circumstances the reaction is complete in about ten minutes); on leaving it over night however the colour was found fully developed in the morning. Samples of ether and of benzol, respectively, were next well shaken with water, allowed to settle, the supernatant liquid pipetted off, and used for making a guaiac solution. Both ether—and guaiac—solutions now behaved precisely as the solutions in the other liquids. Hence traces of water appear necessary for the reaction. Metallic copper therefore appears in the presence of oxygen and water to act upon guaiac resin in precisely the same manner as potato oxidase, and in this respect offers a marked contrast to the other metals examined.

The effect of copper salts. Traces of copper salts have the power of blueing guaiac even more rapidly than metallic copper. If a single drop of a 1 % solution of cupric chloride or sulphate be added to either a solution of guaiac in alcohol or to an emulsion made by adding guaiac tincture to water, an intense blue colour rapidly appears. The depth of colour is out of all proportion to the amount of copper salt added, and is intensified by warmth. The intense blue colour might suggest the formation of a copper salt. That this is not the case, is shown by extracting the blued emulsion with chloroform, and evaporating to dryness. The residue again has all the characters of unaltered guaiac, and on ignition leaves no residue.

Salts of certain other metals however possess the power of blueing guaiac. Amongst these are naturally the powerful oxidizers ferric chloride and potassium permanganate. In the present research freshly prepared solutions of the following salts were used with a negative result:—Pb $(CH_3COO)_2$, $HgCl_2$, $FeSO_4$, $MnSO_4$, $NiCl_2$, $CdSO_4$, and $ZnSO_4$. On the other hand $AgNO_3$ gave a good reaction and $CoCl_2$ a slight one. In no case however except those of $FeCl_3$ and $KMnO_4$ were the results comparable in intensity with those obtained from traces of copper salts, of which the chloride, sulphate, and acetate were tried.

To sum up, both ordinary metallic copper and its salts have a catalytic oxidizing action on guaiac, which so far as examined, appears to be specific for that metal as regards the intensity of the reaction.

THE MODE OF ACTION OF NICOTINE AND CURARI, DETERMINED BY THE FORM OF THE CONTRACTION CURVE AND THE METHOD OF TEMPERATURE COEFFICIENTS.

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(*From the Physiological Laboratory, Cambridge.*)

In recent years there has been a tendency to attribute to a physical rather than to a chemical process, the action of many substances which have an effect upon the organism when given in very minute quantities. In very few cases, however, has the physical view been worked out in any detail. The actions of nicotine and of curari have been investigated by Prof. Langley on the lines of physiological experiment, and he advocates the view that these, and other similar bodies, in producing their specific effects form reversible chemical combinations with certain constituents—"receptive substances"—of the cells. In the following pages I have tested the mode of action of nicotine and curari, on the skeletal muscles of the frog, by mathematical and physico-chemical methods.

1. Mathematical consideration of the curves of muscular contraction and relaxation.

The action of nicotine and of Ringer's solution after nicotine.

The apparatus used for recording the contractions was that designed by Mr Keith Lucas for Prof. Langley. The point of the lever described a vertical straight line on a stationary drum. The muscle used in all cases unless otherwise stated was the *rectus abdominis* of *Rana Temporaria*. It was allowed to relax completely in Ringer's solution under the weight of the lever (about 2·5 gr.) before being immersed in the nicotine solution.

Curves of contraction, recorded on a slowly rotating drum, were obtained by the use of dilute (·00003 %) nicotine. Within the limits of error I found the curves of contraction followed the equation,

$$y = k\,(1 - e^{-\lambda t}),$$

where y is the height of contraction, and t is the time measured from the beginning of the contraction.

The method used for verifying the equation of the curve was as follows:—The ordinates measured *down* from the asymptote (the full height of contraction, $y = k$) were taken at equal intervals of time from the tracing. These should be in geometrical progression.

For example, if AB, CD, EF, GH,... be drawn at equal intervals of time to meet the asymptote in A, C, E, G,... and the curve in B, D, F, H,... then $\frac{AB}{CD} = \frac{CD}{EF} = \frac{EF}{GH} = \ldots$. If the interval of time were n minutes,

$$y_t = k\,(1 - e^{-\lambda t}),$$

$$y_{t+n} = k\,(1 - e^{-\lambda (t+n)}),$$

$$\frac{k - y_t}{k - y_{t+n}} = \frac{ke^{-\lambda t}}{ke^{-\lambda (t+n)}} = e^{\lambda n},$$

which is a constant not depending on t.

The ratios of successive ordinates at equal intervals of time were taken, and found to be practically the same all along the curve. Below are given a few typical results selected at random.

I. Interval 2′.

Ratios of successive ordinates:—·63, ·68, ·77, ·71, ·73, ·71, ·77, ·81, ·76, ·75, ·77, ·76, ·75.

II. Interval 3′.

Ratios of successive ordinates:—·75, ·75, ·90, ·88, ·89, ·79, ·82, ·86, ·89, ·84, ·82, ·85, ·87, ·88.

III. Interval ½′.

Ratios of successive ordinates:—·82, ·81, ·83, ·86, ·85, ·86, ·86, ·88, ·85, ·85, ·86, ·86, ·89, ·89, ·89, ·89, ·89, ·89, ·90, ·90.

IV. Interval 1·5′.

Ratios of successive ordinates:—·83, ·77, ·72, ·71, ·69, ·74, ·75, ·74, ·77, ·74, ·74, ·71.

V. Interval 40″.

Ratios of successive ordinates:—·74, ·77, ·77, ·78, ·78, ·76, ·72, ·77, ·70, ·84, ·78.

VI. Interval 40″.

Ratios of successive ordinates:—·93, ·93, ·94, ·93, ·93, ·93, ·94, ·94, ·94, ·94, ·94, ·94.

Some twenty of such curves were analysed and the same results appeared in all. Thus the curve of nicotine contraction follows the equation,

$$y = k\,(1 - e^{-\lambda t}).$$

The next point to determine was the equation of the curve of relaxation when a *rectus abdominis* muscle which had contracted in dilute nicotine was washed with, and left to relax in, Ringer's solution.

The curve obeyed the equation,

$$y = ke^{-\lambda t}.$$

Below are given a few typical results. The ordinates are here measured from the level to which the muscle finally relaxes after 4 or 5 hours.

I. Time interval 20′.

Ratios of successive ordinates:—·41, ·71, ·84, ·81, ·85, ·80, ·80, ·79, ·82, ·78, ·81, ·81.

II. Time interval 20′.

Ratios of successive ordinates:—·88, ·77, ·70, ·68, ·71, ·70, ·77, ·67.

III. Time interval 25′.

Ratios of successive ordinates:—·74, ·64, ·63, ·69, ·75, ·69, ·69, ·64.

Quite at the end of a relaxation (or contraction) when the quantities to be measured are so small (1 mm. or less) that errors of observation make the ratios irregular, the ratios are omitted. The usual height of contraction is of the order of several centimetres, and exact measurements are easily made.

The question arises, What reasonably simple explanation can we give of the equations $y = k\,(1 - e^{-\lambda t})$, during contraction, and $y = ke^{-\lambda t}$, during relaxation? The answer must in all probability be a simple one: otherwise we should not have the extraordinary coincidence of such simple equations holding for such long periods of time. Two possible solutions can be advanced. The form of the curve is due either (*a*) to the gradual diffusion of the nicotine inwards, or (*b*) to a gradual combination (chemical or otherwise) between nicotine and some substance in the muscle.

(*a*) Let us suppose that the height of contraction, y, is directly proportional to $(N - M)$, where N is the amount of nicotine that has diffused in at any time, and M is the minimum amount that will cause any contraction at all. By N_0 is meant the concentration of nicotine in the bathing fluid.

Then the rate of diffusion inwards is proportional to the difference between the concentrations inside and outside.

i.e. $\frac{dN}{dt}$ is proportional to $(N_0 - N) = \lambda (N_0 - N)$,

i.e. $$\int_0^N \frac{dN}{N_0 - N} = \int_0^t \lambda dt,$$

i.e. $$\log \frac{N_0 - N}{N_0} = -\lambda t,$$

i.e. $$N = N_0 (1 - e^{-\lambda t}).$$

Now, by hypothesis y is proportional to $N - M$,

i.e. $$y = k' (N_0 - M - N_0 e^{-\lambda t}) = k' (N_0 - M)(1 - e^{-\lambda t + \log \frac{N_0}{N_0 - M}}),$$

which becomes

$$y = k (1 - e^{-\lambda t}),$$

if we measure the time from a time $\frac{1}{\lambda} \log \frac{N_0}{N_0 - M}$ after the muscle was immersed in nicotine.

For the relaxation we have, when the concentration of nicotine outside is zero,

$$-\frac{dN}{dt} = \lambda N.$$

This gives $y + k'M = k'N_0 e^{-\lambda t}$, and $y + k'M$ is the height above a certain line very nearly at the final position of relaxation.

(*b*) Let us, on the other hand, suppose that nicotine combines, for example chemically, with some constituent A of the muscle, and that the height of contraction y is directly proportional to

$$(NA) - M,$$

where (NA) is the amount of the compound formed by nicotine with that constituent, and M is the least amount necessary for any contraction to occur.

It is quite reasonable to suppose that diffusion is so rapid as not sensibly to affect anything but the initial shape of the curve. We have

$$N + A \rightleftarrows NA.$$

The action is undoubtedly reversible as complete relaxation in Ringer's solution can be obtained.

As Arrhenius points out[1] the ordinary laws of chemical dynamics can be taken as true for heterogeneous, as well as for homogeneous media, provided that diffusion can go on with sufficient rapidity.

[1] *Immuno-Chemistry*, p. 142. 1907.

Let the velocity constants for the action ⟶ be k and ⟵ be k': we have:

$$\frac{d(NA)}{dt} = k(N)(A) - k'(NA).$$

Now $(A) + (NA) = X$, a constant, the number of molecules of the substance A present.

$$\therefore \frac{d(NA)}{dt} = -(k' + kN)(NA) + k(N)X,$$

i.e. $$\int_0^{(NA)} \frac{-d(NA)[k' + k(N)]}{k(N)X - [k' + k(N)](NA)} = \int_0^t -dt[k' + k(N)],$$

which gives

$$(NA) = \frac{k(N)X}{k' + k(N)}\{1 - e^{-[k' + k(N)]t}\}.$$

This, as above, on measuring the time from a suitable place, becomes:

$$y = \mu\left\{\frac{k(N)X}{[k' + k(N)]} - M\right\}\{1 - e^{-[k' + k(N)]t}\}.$$

(N) is here the concentration of nicotine both inside and outside the muscle, supposed to have become equal in a comparatively short time.

For the relaxation we have

$$y + M\mu = \mu(NA)_0 e^{-k't},$$

where $(NA)_0$ is the initial amount of NA present.

To decide between the hypotheses (a) and (b) two methods may be used, (A) and (B) below.

A. The total height of contraction should be, according to (a) (the physical hypothesis) proportional to $N - M$, and according to (b) (the chemical hypothesis) proportional to $\frac{N}{k' + kN} - M$.

To decide this a *rectus abdominis* muscle, lightly weighted, was allowed to stay successively in the following concentrations of nicotine, until fully contracted in each.

Strength of nicotine	$N = \cdot000008\ \%\times 1$,	$\cdot000008\ \%\times 1{\cdot}96$,
Height of contraction	$y = 4$ mm.,	11·9 mm.,
	$N = \cdot000008\ \%\times 2{\cdot}89$,	$\cdot000008\ \%\times 3{\cdot}77$,
	$y = 16{\cdot}7$ mm.,	20·3 mm.,
	$N = \cdot000008\ \%\times 4{\cdot}63$,	$\cdot000008\ \%\times 6{\cdot}23$,
	$y = 21{\cdot}6$ mm.,	24 mm.

Here the product $(a - y)(b + N)$, where $a = 33{\cdot}8$, $b = 1{\cdot}54$, has the following values:

76·0, 76·6, 75·5, 72·0, 75·3, 75·5,

(N is taken as 1 when the real value is ·000008 %). Hence $(33{\cdot}8 - y)(1{\cdot}54 + N)$ is almost exactly constant and equal to a mean value 75·2.

This becomes $$y = 33{\cdot}8 - \frac{75{\cdot}2}{1{\cdot}54 + N},$$

or $$y = \frac{48{\cdot}8N}{N + 1{\cdot}54} - 15.$$

This is exactly of the form required of the height of contraction,

$$y = \frac{N}{k' + kN} - M,$$

and is very strong evidence in favour of the hypothesis of a combination between nicotine and some constituent of the muscle.

B. The physical hypothesis leads to an equation

$$y = \mu'(1 - e^{-\lambda t}),$$

where λ, and therefore $e^{-\lambda}$, does not depend on N, the strength of nicotine in the solution: the hypothesis of combination to an equation

$$y = \mu'(1 - e^{-(k' + kN)t}).$$

Experimentally the equation of the curve is found to be

$$y = \mu'(1 - e^{-at}).$$

e^{-a} is given below for several different pairs of contractions. Two halves of the same *rectus abdominis* were treated with different concentrations of nicotine, and the e^{-a} of each resulting curve calculated. If the action were physical in nature e^{-a} should be identical for the two different concentrations.

I.	(*a*)	One half in ·0001 %,	$e^{-a} = {\cdot}50$.
	(*b*)	Other half in ·000066 %,	$e^{-a} = {\cdot}57$.
II.	(*a*)	One half in ·0001 %,	$e^{-a} = {\cdot}47$.
	(*b*)	Other half in ·000066 %,	$e^{-a} = {\cdot}64$.
III.	(*a*)	One half in ·00012 %,	$e^{-a} = {\cdot}51$.
	(*b*)	Other half in ·00004 %,	$e^{-a} = {\cdot}70$.
IV.	(*a*)	One half in ·0001 %,	$e^{-a} = {\cdot}62$.
	(*b*)	Other half in ·00005 %,	$e^{-a} = {\cdot}67$.
V.	(*a*)	One half in ·0008 %,	$e^{-a} = {\cdot}33$.
	(*b*)	Other half in ·00012 %,	$e^{-a} = {\cdot}60$.

Thus in every case the e^{-a} of the lower concentration is greater than the e^{-a} of the higher concentration. This inequality is not explicable on a purely physical hypothesis of diffusion. According to the chemical hypothesis

$$e^{-a} = e^{-(k' + kN)},$$

Increasing N therefore increases a, and therefore decreases e^{-a}. Hence the fact that at the lower concentration e^{-a} is the greater is exactly as it should be on the hypothesis of a chemical combination.

One or two accessory points may be noticed. In finding the equations of the curves one of two cases usually arose.

(*a*) If the muscle were quite fresh the curve rose initially much faster than required by the equation to the greater part of the curve.

(*b*) If it were kept 24 hours in Ringer's solution, or contracted in nicotine once, and then relaxed again in Ringer's solution, it contracted initially rather slower than required by the greater part of the curve.

The explanation of (*b*) seems to be that diffusion into the fibres was not instantaneous, and therefore initially, with less nicotine inside, the rate of contraction was less than the calculated rate.

The explanation of (*a*) I believe to be as follows. The equation of the curve in these cases can be written,

$$y = k(1 - e^{-\lambda t}) + k'(1 - e^{-\lambda' t}),$$

where λ is something like 10 times as great as λ'. This means that two separate fibres are contracting, one many times faster than the other. The contraction of the first is complete in (say) a minute, while the second is only complete in ten. This *may* be due, not to two different fibres, but to two different types of contraction in the same fibre. There is in fact evidence[1] in some muscles of several different fibres, contracting at different rates.

The usual relaxation in Ringer's solution is also generally much more rapid at first than would be expected. The curve of relaxation can in these cases again be analysed into two exponential curves. In one case a muscle was left to contract *all day* in ·00003 % nicotine and then allowed to relax in Ringer's solution: only one half of the total relaxation occurred and the initial relaxation followed the equation as well as the later. Apparently here there was a pure case of the *slow* fibres alone relaxing.

Thus the activity of the quickly contracting and relaxing fibres is much sooner abolished in dilute nicotine than the activity of the slower working fibres. The quick fibres seem to pass from their tonic contraction in dilute nicotine to a state of permanent shortening. The slow fibres survive much longer.

[1] Cf. Langley, *op. cit.* p. 248. 1909.

The action of curari.

Curari and nicotine are "antagonistic" drugs. Prof. Langley[1] has given reasons for supposing that curari, like nicotine, combines with the "receptive substance" of muscles. He also shows that the curari compound, whose existence causes no contraction, is more stable than the nicotine compound.

I have applied an analysis of the curves of relaxation under the action of curari, similar to that given above for the curves of relaxation in Ringer's solution after nicotine.

I give details of two experiments.

I. A *rectus abdominis* muscle was immersed in ·0006 % nicotine and then in ·0006 % nicotine + ·05 % curari. A complete relaxation occurred from a height about ·8 cm. in a muscle of about 2·2 cm. This relaxation took 25 minutes to reach completion, the first half taking 8·5 minutes. The equation of the curve was approximately $y = ke^{-\lambda t}$.

II. A muscle was put in ·0001 % nicotine, and then in ·0001 % nicotine + ·0006 % curari. The heights above the asymptote at equal intervals of time, 40 minutes, are in the ratios

·76, ·78, ·80, ·78, ·80, ·84, ·76, ·77.

The ratios are therefore constant, and the curve of relaxation is $y = ke^{-\lambda t}$.

This may have, as before, two possible explanations. On a hypothesis of combination the form of the curve does not enable us to decide whether the curari is combining with some distinct substance, or whether it is turning nicotine out of some combination, and replacing it. The latter can be proved to necessitate a curve, $y = k(1 - e^{-\lambda t})$, though the mathematical analysis proving it is complicated and unnecessary here.

2. The temperature coefficients of the contraction caused by nicotine and of the subsequent relaxation caused by curari.

Nicotine.

It is well known that Arrhenius and others have shown that the temperature coefficient is much higher in chemical than in physical processes. Although the difference is not absolutely to be relied on as a distinguishing character, it gives evidence of considerable weight in a case of this kind.

[1] This *Journal*, xxxix. p. 235. 1909, and other papers.

The constant calculated was that employed in most works on Physical Chemistry[1], and may be obtained from the equation,

$$v_2 = v_1 e^{\frac{\mu}{2}\frac{T_2 - T_1}{T_2 T_1}},$$

where v_2 and v_1 are the velocities of contraction at temperatures T_2 and T_1. My reasons for using the constant μ are, (1) it is more general than any other, (2) it can be compared with the value of μ found by Arrhenius[2] for many biological actions. $\mu = 17{,}850$ corresponds to a velocity at 17° C. three times that of the same action at 7° C.

The method was as follows:

A *rectus abdominis* muscle from a large female frog was dissected out and divided carefully down the tendon in the middle line. The two parts, even if not of the same size and strength, are probably of almost exactly the same quality as regards susceptibility to stimulation and velocity of contraction. The two halves were immersed in dilute nicotine at temperatures T_2 and T_1 respectively, and the equations of the resulting curves were obtained.

Suppose that the equations were:

$$y = k_2 (1 - 10^{-\lambda_2 t}), \text{ at temperature } T_2,$$

$$y = k_1 (1 - 10^{-\lambda_1 t}), \text{ at temperature } T_1.$$

Then we wish to know the ratio of the times in which the two muscles reach the same percentage of their total contractions. The equations may be written

$$y_1 = k_2 (1 - 10^{-\lambda_2 t}) \quad \text{and} \quad y_1 = k_1 (1 - 10^{-\lambda_2 \frac{t\lambda_1}{\lambda_2}}).$$

Then the first one is at the same stage of contraction (say any given percentage of its total contraction) at time t, that the second is at time $t\frac{\lambda_2}{\lambda_1}$, for then

$$y_1 = k_1 (1 - 10^{-\lambda_2 t \frac{\lambda_2}{\lambda_1}\frac{\lambda_1}{\lambda_2}}) = k_1 (1 - 10^{-\lambda_2 t}),$$

and hence

$$\frac{y_1}{k_1} = \frac{y_2}{k_2}.$$

Hence

$$\frac{v_2}{v_1} = \frac{\frac{1}{t}}{\frac{1}{t\frac{\lambda_2}{\lambda_1}}} = \frac{\lambda_2}{\lambda_1}.$$

[1] Cf. Nernst. *Theoretical Chemistry*, p. 673 *et seq.*

[2] Arrhenius. *Immuno-Chemistry*, 1907.

Hence $$\lambda_2 = \lambda_1 e^{\frac{\mu}{2}\frac{T_2 - T_1}{T_2 T_1}},$$

and from this μ can be calculated. This method was later replaced by finding the times in which the two halves reached a given proportion (·6 or ·8) of their total contraction. This was far easier, but inasmuch as it depends on only one measurement, and the former depends on taking the mean of many, it is less rigid. However as the results obtained from the simpler method seemed to agree very well with those obtained from the more rigid, it was uniformly adopted in the later calculations.

I. Left half in ·00003 % nicotine at 19·65° C.:

$$y = 22{\cdot}3\ (1 - 10^{-\sigma \cdot 1190}).$$

Right half in ·00003 % nicotine at 8·3° C.:

$$y = 30{\cdot}9\ (1 - 10^{-\sigma \cdot 0311}).$$

$$\frac{v_2}{v_1} = \frac{\cdot 1190}{\cdot 0311} = 3{\cdot}82,$$

$$\mu = 19{,}550.$$

II. $T_1 = 6{\cdot}6°$ C., $T_2 = 16{\cdot}3°$ C.

$$\mu = 17{,}900.$$

III. $T_1 = 6°$ C., $T_2 = 20{\cdot}4°$ C., Nicotine ·001 %.

$$\mu = 16{,}700.$$

IV. ·00004 % nicotine. $\mu = 14{,}220.$

V. ·001 % nicotine. $\mu = 16{,}700.$

VI. ·0006 % nicotine. $\mu = 19{,}000.$

The small variations in the temperature coefficient are probably due to differences in the two halves of the same muscle. The assumption that the two halves are the same in quality is not entirely justified except by taking the mean of several observations. The mean value of μ is

$$\mu = 17{,}340.$$

This is far too high for the action to be purely physical. For example for diffusion through a membrane the effect of change of temperature is given by

$$\mu = 289 \text{ (approx.)}.$$

If it were not that the hypothesis of physical diffusion alone is not sufficient to explain the considerations given in Section 1 of this paper, the high value of μ might be explained as the temperature coefficient of the permeability of the walls of the muscle fibres. It is quite possible that the chemical nature of the walls varies with the tempera-

ture, and that the permeability, and consequently the rate of diffusion inwards, have a very high temperature coefficient[1]. However, since the hypothesis of physical diffusion is insufficient, we must turn to that of a combination between nicotine and a "receptive substance." The high value of μ then shows that this combination is of an ordinary chemical nature.

In the preceding observations the percentage of nicotine was sufficiently small to give the specific action of the poison, *i.e.* an action on the "receptive substance." When the percentage of nicotine passes a certain limit the general muscle substance is also affected, and according to Prof. Langley two actions on the general muscle substance are to be distinguished, viz. a stimulating action soon over, and a very slow action causing rigor. I have not attempted to determine separately the temperature coefficients of these reactions of the general muscle substance, but I have in a few cases determined the temperature coefficients of the total initial contraction caused by strong nicotine. The curves were no longer exponential curves, but might have their equations written $y=f(t)$, and $y=f(\lambda t)$: in other words the curves might be made identical by plotting one of them with a larger time scale. The constant λ, or the ratio of the times to equal heights in the two contractions, gives us the temperature coefficient. The temperatures were in all cases about 5° C. and about 18° C., except for the secondary rise of the sartorius (No. 4) when they were 16° and 26° C.

1. *Rectus abdominis*, nicotine ·1 %. $\mu=14,500$.

2. *Rectus abdominis*, nicotine 1 %. $\mu=15,240$.

3. *Sartorius*, nicotine 1 %. The primary contraction was taken, *i.e.* the rise which occurs before the secondary (rigor) rise began.

Exp. i. $\mu=14,000$.
Exp. ii. $\mu=14,900$.
Exp. iii. $\mu=15,000$.

4. *Sartorius*, nicotine 1 %. Contraction taken after the secondary rise was well established.

Exp. i. $\mu=11,500$.
Exp. ii. $\mu=23,500$.

It will be seen that the temperature coefficient of the primary contraction caused by strong nicotine is much the same as that caused by very dilute nicotine: again therefore we have evidence that nicotine is acting by means of a chemical combination.

[1] It is possible that the physiological permeability of a membrane depends on its power of combining with the substance it transmits. In which case the rate of diffusion inwards would increase rapidly with the temperature.

The action of curari.

I. Two halves *A* and *B* were immersed (i) in ·0006 % nicotine, and then (ii) in ·0006 % nicotine + ·05 % curari.

A at 8·2° C., *B* at 17·2° C.

The ratio of the rates of relaxation was 1·50.

$\mu = 7{,}400.$

II. In a similar experiment the ratio, for a difference of 14° C., was 1·63.

$\mu = 5{,}700.$

The action of curari therefore seems to be by means of a chemical combination. The value of μ is lower than for nicotine contraction. This may be simply a peculiarity of the combination between curari and "receptive substance," or it may be due to the mechanical viscosity of the contracted muscle which does not relax as fast as the curari compound is formed.

In conclusion I should like to express my thanks to Prof. Langley for his kindness in helping me, both by criticism and advice: and to Mr C. G. Darwin of Trinity College, for his aid in my analysis of the curves.

Conclusions.

1. The curve of contraction of a *rectus abdominis* muscle in dilute nicotine follows an equation

$$y = k(1 - e^{-\lambda t}),$$

where y is the height of contraction, t is the time, k and λ are constants for that particular curve.

2. The curve of relaxation of a *rectus abdominis* muscle, which has contracted in dilute nicotine, and is then placed in Ringer's solution, or in a solution of curari, follows the equation

$$y = ke^{-\lambda t}.$$

3. Two hypotheses to account for this are considered:

(*a*) that of a gradual diffusion of the drug in or out,

(*b*) that of a gradual combination of the drug with some constituent of the muscle.

Two methods, (*A*) comparison of total heights, (*B*) comparison of velocities of contraction, at the same temperature with different

strengths of nicotine, point to the hypothesis of a combination as the only possible one.

4. The temperature coefficient μ, of the velocity of contraction, shows that the combination between nicotine or curari and the combining constituent of the muscle is of an ordinary chemical nature. This combination is a reversible one between two molecules

$$N + A \rightleftarrows NA,$$

and the height of contraction at any moment is proportional to $(NA - M)$, where NA is the amount of the compound formed at that moment, and M is the minimum amount necessary to cause any contraction. Curari also combines chemically with a constituent of the muscle.

5. Muscular contractions of either the *sartorius* or *rectus abdominis* in very widely different concentrations of nicotine (·00003 % to 1 %) give values of μ never very far from 16,000. These values lie very close to those given by Arrhenius for many biological actions. This corresponds to a velocity at 17° C. about 2·8 times that at 7° C. The μ of the action of curari corresponds to a velocity at 17° C. about 1·5 times that at 7° C.

6. Evidence for the existence of two (or more) different types of fibres (or contractions) in the same muscle is given by a mathematical analysis of the curves. The quicker moving fibre (or contraction) is destroyed much sooner than the slower, if left in dilute nicotine and Ringer's solution.

THE EFFECT OF TEMPERATURE ON THE DISSOCIATION CURVE OF BLOOD.

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(*From the Physiological Laboratory, Cambridge.*)

In the course of investigations carried on by one of us in association with Dr Camis[1], it was observed that there was an appreciable difference between the dissociation curves of rabbits' blood at 37° C. and 41° C. respectively in the sense that, at a given tension, the saturation of the hæmoglobin with oxygen was less at the higher temperature. These experiments were carried out without any special precautions as to CO_2 tension, they were not published therefore but were sufficiently suggestive to make it desirable to attempt a more vigorous investigation of the effect of temperature on the dissociation curve of blood: the more so as there is very little known about the subject.

Historical. Paul Bert[2] found that blood was about 90 % saturated at an oxygen tension of 15 mm., the temperature being that of the room, whilst it was only 50 % saturated at the same tension and at the body temperature; these results also are given irrespective of carbonic acid tension and might have been due to some variation in the CO_2 rather than to the assigned cause.

In 1889 Hüfner[3] made the same observations on the subject which tend in the same direction.

Observations with solutions of hæmoglobin. Our first experiments were made not with blood but with hæmoglobin solutions. The methods were in all respects those described by Barcroft and Camis.

The hæmoglobin was prepared by Bohr's method, *i.e.* by crystallising the pigment from the washed corpuscles (dog) with ether, redissolving the crystals and getting rid of the ether by shaking. The dissociation curve was investigated at four temperatures, 14° C., 26° C., 32° C. and 38° C. In all cases except that of 32°, more than one solution was

used for the investigation. The results obtained at 15° C. were very different from anything which had previously been observed by us, but they were identical in the case of the three solutions which we used. The results are shown in Fig. 1 Curve IV. The general nature of the curve indicates the much greater affinity of hæmoglobin for oxygen at the lower temperature. Curves III and II (26° C. and 32° C.) do not need special description so far as the experimental investigation is concerned. Curve I is Bohr's[4] curve for 38° C. which will be seen to correspond very closely with the determinations which we made at that

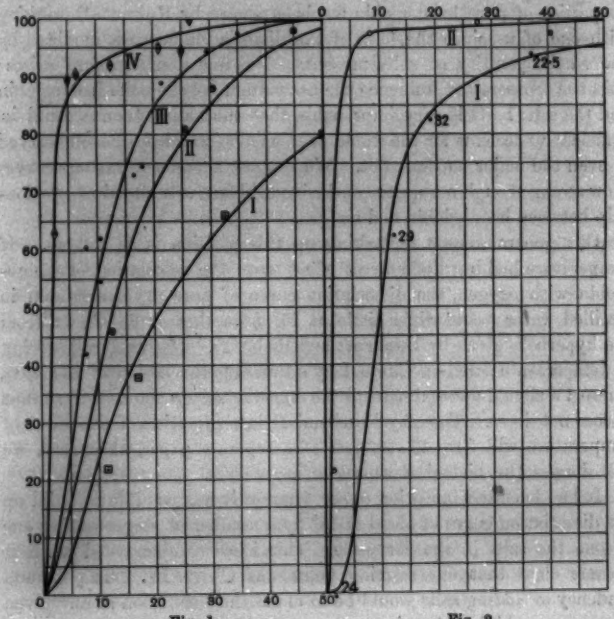

Fig. 1. Fig. 2.

Fig. 1. Dissociation curve of Hb dissolved in distilled water. Tension of oxygen plotted horizontally, percentage saturation vertically. Curves I, II, III, IV correspond to 38°, 32°, 26° and 14° C.

Fig. 2. Dissociation curve of Hb dissolved in ·9 % KCl. I at approximately 25 mm. tension CO_2, II at 0 mm. tension CO_2. The numbers represent the actual CO_2 tensions of the determinations.

temperature—a correspondence which has already been emphasised by Barcroft and Camis.

The great difference between the dissociation curves at 38° C. and at 15° C. raises a number of interesting points which it may be well to state briefly.

1. If the solution could be regarded as a pure solution of hæmoglobin in the unimolecular condition, the large temperature coefficient would be conclusive proof that the relation of oxygen to hæmoglobin was a chemical one, and not a physical one as supposed by W. Ostwald[9].

2. That the solution cannot be regarded as representing the properties of pure hæmoglobin has been shown by Roberts[8] working with one of us, since the form of the dissociation curve is modified by the small quantity of salts present. The question therefore arises; does the temperature influence the association between the hæmoglobin and the salts? It is very improbable that such an influence could be sufficient to account for the whole phenomenon which we have observed or even the major portion of it. We suggest therefore that our curves show strong though not quite conclusive evidence of a chemical relationship between hæmoglobin and oxygen.

Our general reason for taking up this position is as follows. If temperature had but little direct effect upon the association of hæmoglobin with oxygen, the dissociation curve of pure oxyhæmoglobin in distilled water would differ little at 14° from that at 38° C., *i.e.* from the hyperbola given by Barcroft and Roberts. If therefore lowering of temperature acted on Curve I by decreasing the action of the salts, Curve IV should approximate to the hyperbola $xy = 800$ as a limit, but it does not do so. The physico-chemical interpretation of the effect of temperature will form the subject of a separate paper, at present we will discuss the biological aspect of our curves.

It had been our intention to see whether it was possible to build up the dissociation curve of blood at 15° by a number of stages adding one by one the salts in the corpuscle. This idea we abandoned when it became clear that our starting point was Curve IV. The general tendency of adding salts would be to make the saturation at any given pressure even higher than in the hæmoglobin curve. But the curve as it stands leaves little room for this, there seemed therefore to be but slender prospect of getting results which diverged from Curve IV by an amount that we could appreciate with certainty.

We determined therefore to try the salt which would give the maximum divergence, namely potassium chloride. A hæmoglobin

solution made up in 0·9 % KCl (isotonic with 0·72 NaCl) yielded, at 15° C., a dissociation curve which is clearly even more acute than that of hæmoglobin in water at the same temperature. This is shown by Fig. 2. At a tension of 8·5 mm. the solution of Hb in KCl is virtually saturated whilst that in water falls short of saturation by an appreciable amount.

Finding that it was impossible in the nature of things to produce any great change in the curve by the addition of substances which raised the saturation at a given tension we turned our attention in the opposite direction and determined the effect of carbonic acid on the solution of hæmoglobin in potassium chloride. This is shown in Curve I Fig. 2. A comparison of Fig. 2 with Fig. 4 in the paper of Barcroft and Camis, will show that while it is not possible to compare with mathematical precision the effects of CO_2 upon two curves which differ so much, it is possible to say in a general sense that the effect of CO_2 in dissociating the oxygen from hæmoglobin in KCl solution at 15° and from blood at 38° C. is of the same order of magnitude.

Furthermore whilst the effect in the two cases may be regarded as of the same order of magnitude absolutely, relatively CO_2 is far more potent at 15° than at 38°, *i.e.* the amount of hæmoglobin reduced by the CO_2 bears a much bigger proportion to the total amount of reduced hæmoglobin.

The biological significance of this fact is probably very great, for it means that the potent factor in removing the oxygen from the blood of poikilothermic animals when the temperature is low is carbonic acid and not reduced oxygen pressure, and it is only in the most extreme cases that reduced oxygen pressure can have an appreciable effect.

The elimination of carbonic acid from the frog has been studied in a very exhaustive way by Krogh[7] who states that under most circumstances the output of carbonic acid from the lung of the frog is almost *nil* (the output from the skin being always considerable). After putting forward the thesis that CO_2 is actively prevented from escaping from the lung epithelium, he makes the following statement. "Of the biological importance of the inhibition found, which must at first sight appear quite paradoxical, I can give no absolutely certain explanation.... Suppose the carbonic acid might diffuse freely through the lungs the tension of carbonic acid in the blood which beforehand is remarkably low would fall to an extremely small value. This may reasonably be supposed to be fatal to the animal." He then suggests, on the basis of Schierbeck's work, that the CO_2 is present for the purpose of stimulating ferment action.

Without attempting any criticism of Krogh's experiments we may point out that the data which we have obtained supply a very cogent reason why carbonic acid should be present at an appreciable tension in the blood of cold-blooded animals, for otherwise it would be difficult to see how the corpuscle would lose its oxygen in the capillary.

Moreover these results are instructive in connection with the distribution of hæmoglobin throughout the animal kingdom.

On the authority of Gamgee[68, 69] hæmoglobin may be said to exist in the invertebrata in two rôles.

(1) Diffused certain muscular or nervous cells of Lymnæus, Paludina littorina, Patella, Chiton, Aplysia, etc. among the molluscs, and of Aphrodite and certain of the nemertine worms. In these cases it would probably function as an oxygen store, this would appear to be the less specialised and more primitive condition. The difference between what we have called a "store" and a true "respiratory pigment" should be made clear.

In true respiration the oxygen is taken up by the hæmoglobin at a place where the oxygen tension is high and transferred to another position at which the tension is low; there the gas is given out and thus a constant supply of oxygen is conveyed to the tissue.

In the cases under consideration the hæmoglobin is stationary; and, as clearly it can only be exposed to one tension at a time, it will retain its oxygen at a constant saturation unless some change takes place in its environment. Presumably under normal circumstances it has access to fluid with sufficient oxygen to saturate it (this in the absence of CO_2 would mean a tension of only about 7 mm. of mercury, or a concentration which would be but 5 % of that of oxygen in water shaken with air). Under altered circumstances in which either oxygen is withheld from the cell or the activity of the cell is such as to make it require an extra quantity of oxygen, the cell could withdraw oxygen from the "store."

The step from a muscle which has hæmoglobin in the matrix in which the fibrils are laid down to a heart which has hæmoglobin in the perivascular fluid is not a very great one and marks the stage of a "floating store." Clearly the floating store has many advantages over a fixed one, but the conditions under which it exists are not by any means favourable to the giving up of oxygen to the tissue.

In the worm and leech there is of course a definite circulation of the hæmoglobin. In these forms there is an approximation to true respiration inasmuch as the blood is supposed to take up oxygen in the skin and transfer it to the tissues[68]. The conditions in the leech are clearly favourable to a true respiration, for not only does the blood go

to the surface but the integument is extremely tough and it is probable therefore that a carbonic acid tension may be attained inside the animal, sufficient to dissociate the oxygen more rapidly from the hæmoglobin than would otherwise be the case. In this respect the effect of the first few millimetres of CO_2 tension is relatively very great.

The vertebrata are on the whole large organisms enclosed in a very definite integument which presents a small surface relatively to the size of the animal. The opportunities therefore for the escape of carbonic acid are (relatively to the production) small and therefore an appreciable CO_2 tension exists in the tissues and this fact seems to be the most potent one in providing a mechanism for the tissue respiration at low or even temperate body temperatures.

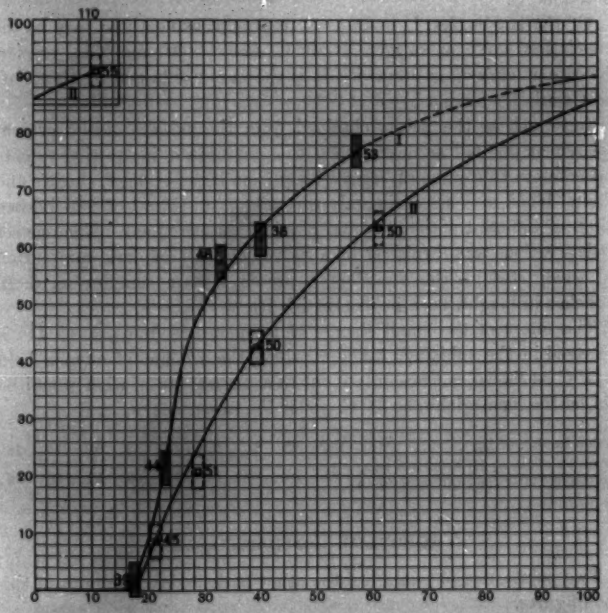

Fig. 3. Rabbits' blood. Percentage saturation with oxygen plotted vertically—oxygen tension horizontally. Curve I at 38° C.; curve II at 41° C. The figures beside the points determined represent the CO_2 tensions. The curves represent approximately 40 mm. tension. The rectangular areas represent the experimental error.

Observations upon blood. The results which we had obtained up to this point had been so striking that we thought it worth while to test the effect of a rise of temperature such as might take place in an acute fever, upon the normal dissociation curve of blood with about 40 mm. tension of CO_2.

Before attempting to investigate human blood in this respect we made an observation upon rabbits' blood at 36 and 41° C. respectively. The two curves are given in Fig. 3, together with the actual determinations. These curves show that at oxygen tensions of between 80 and 15 mm. the blood parts with its oxygen much more readily at the higher temperature.

Fig. 4 shows the result of observations made upon human blood at 36 and 41° C.

Of the two curves, II is drawn to represent as nearly as may be the dissociation curve of oxygen at 41° C. and at 40 mm. CO_2 tension. The case of curve I is otherwise, it is the curve obtained by Barcroft and Camis for human blood at 37°.

Inasmuch as the points obtained from the subject of the present research at 36° fall so nearly upon this curve, it is interesting to note that whereas this curve diverges widely from those of dogs', sheep's and rabbits' blood it is approximately correct for the blood of two human beings of different nationality and physical temperament. The one set of observations was furnished by a native of Italy of dark complexion and small stature, the other by a person of fair complexion, 5 ft. 10 inches in height, born in the British Isles.

The effect of the small change of temperature from 36° to 41° is expressed in the subjoined table, in which the amount of oxygen held at 41° C. is shown, not absolutely but as a percentage of what would be held by the hæmoglobin at 36° C. under the same tension.

Tension in mm. of Hg	100	70	60	50	40	30	20	19	18
Saturation of Hb at 41° expressed as a percentage of the saturation at 36°	99	95	90	84	77	69	54	26	14

Theoretical Considerations.

Two points, one physiological the other pathological, arise out of the above figures.

1. *Tissue respiration during muscular exercise.* In man the temperature is known to rise during violent exercise. This rise is due to the heat production of the muscles being greater than the loss of

heat from the body. In the muscle itself the rise of temperature has a twofold aspect.

(*a*) The local temperature is higher than the general temperature.

(*b*) The general temperature is abnormally high.

These effects will be reflected in the dissociation curve. Taking the general rise of temperature first. A temperature of 41° C. is no doubt excessive for the general temperature of the body even during violent exercise, though so far as we know the recorded effects of exercise have been taken after, and not during the exercise, therefore the general change in the dissociation curve would be somewhat less than that

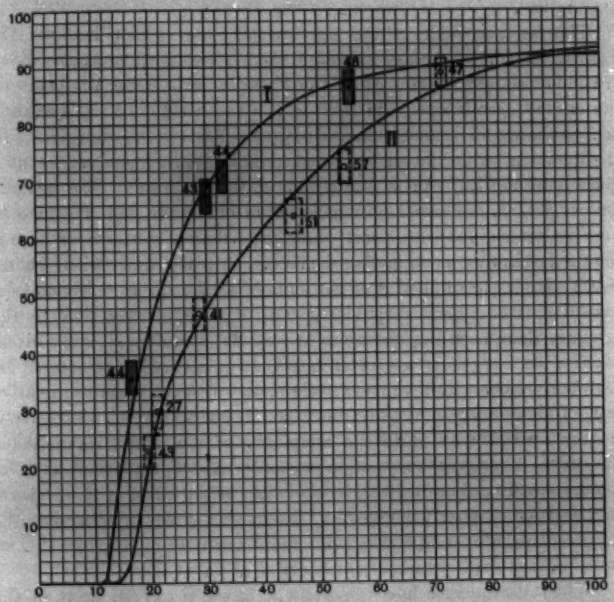

Fig. 4. Human blood. Percentage saturation with oxygen plotted vertically; oxygen tension in mm. horizontally. Curve I is that of Barcroft and Camis for human blood at 37°, the determinations near it were made at 36° with the blood under investigation. II at 41° C. The figures attached to the determinations indicate the CO_2 tensions. The curves represent approximately 40 mm. CO_2 tension. The rectangular areas represent the experimental error.

represented in Fig. 4. Nevertheless it would be sufficient to cause the oxygen to be yielded to the muscle more readily than under normal circumstances.

When the local effect is superimposed upon the general effect it is by no means impossible that temperatures approaching 41° may be reached in the actual muscles in man. So that the blood may be heated up in the contracting muscle to such an extent that the curve in Fig. 4 represents the properties of the blood in the muscle without great error.

One point must be borne in mind, namely that while we have treated the problems under our consideration as statical they are in reality kinematical.

This is true both of the physical processes which underlie the dissociation curves themselves and of the physiological processes which are influenced by them. From the chemical side a certain point on the curve depends upon the ratio of the velocities of the dissociation and association of hæmoglobin and oxygen—from the physiological side, the question before the muscle is not: can the muscle get oxygen at a given tension? but can it get oxygen at a given rate? The demand for oxygen, *i.e.* the increase in metabolism of muscle, can of course be directly determined: the point which immediately concerns us here is the mechanism for meeting that demand. Increased quantities of blood pass through the capillaries of the muscle as the direct result of local vaso-dilatation. Each corpuscle spends a much shorter time in the capillary than when the muscle is at rest and therefore it must divest itself of its oxygen at a greater rate than is normally necessary. Do the observations which we have made show any light upon the relative ratio of dissociation of oxygen from blood at 36° and 41° C. respectively. It is not possible, owing to the complexity of the factors which shape the curves, to answer the question precisely. A commencement however has been made in this direction by the study of the effect of temperature on a solution of pure hæmoglobin. From this it will appear that the oxygen dissociates from hæmoglobin about twice as rapidly at 41° C. as at 36° C. and about 50 times as rapidly as at 14° C.

When we consider how important a factor is oxygen want in the production of muscular fatigue, the beneficent effect of even a small increase of the body temperature will easily be appreciated.

2. *Tissue respiration during fever.* The reaction of hæmoglobin to a rise of temperature is also apparent during fever. Every tissue in the body is in an exalted state of metabolism, *i.e.* it wants more

oxygen than normally, nothing could be more appropriate than that the hæmoglobin should yield its oxygen more rapidly. The argument which we have set forth may be applied also to local inflammations. At the stages in which the corpuscle goes more rapidly through the capillary a general rise of temperature is beneficial to the inflamed part. The reaction of the thermogenic centre to the toxine thrown into the blood is protective in this, as well as in other ways.

Summary.

1. Dissociation curves of undialysed hæmoglobin solution in water are delineated at 14°, 26°, 32°, 38° C., in KCl at 14° in the presence and absence of CO_2.

2. In the case of the latter solution the presence of CO_2 is relatively a much more potent influence in the reduction of hæmoglobin than in the removal of oxygen.

3. In the light of the above facts, it is suggested that in certain low forms of animal life in which hæmoglobin exists in the substance of the nervous and muscular systems it is retained as a "store" from which the oxygen can be withdrawn in cases of extreme need which might result either from partial asphyxia or great functional activity; by a transition in which the hæmoglobin is placed in the fluid round the organ, a functional respiratory circulation of hæmoglobin might be evolved in forms in which the conditions for the oxygen to be discharged to the tissues are favourable. Such favourable conditions are rise of temperature and rise of CO_2 tension.

4. The great influence of temperature on the dissociation curve is evident in blood no less than in hæmoglobin. This is apparent in the dissociation curves of blood in the rabbit and man at 36° and 41°. During muscular exercise the rise of temperature in the muscles is sufficient to produce an appreciable change in the rate of dissociation of oxyhæmoglobin (in a pure solution of hæmoglobin the rates of dissociation at 36° and 41° C. are as 1 : 1·7), this is significant in relation to the more rapid passage of the corpuscle through capillary of the active organ. In fever also and in inflammation where the whole or a portion of the organism requires oxygen at a more rapid rate than usual, the rise of body-temperature helps in enabling the blood to meet the demand put upon it and is in fact the counterpart of vascular dilatation.

The cost of this research has been defrayed in part by a grant from the Royal Society.

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WATER RIGOR IN FROG'S MUSCLE.

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Formation of lactic acid in water rigor.

It was shown by du Bois Reymond[1] that in water rigor, as in the other forms of rigor which had been investigated, frog's muscle becomes acid. du Bois Reymond left the muscle in water for an hour. It is not necessary, however, to keep the muscle in distilled water so long as this. I have constantly found that small muscles which have been in distilled water for fifteen or twenty minutes are distinctly more acid to litmus than controls which have been for the same length of time in a moist chamber or in Ringer's fluid[2].

To determine whether the acid found in this case is lactic acid, as in the case of heat rigor and death rigor, I have made use of the thiophene test for lactic acid, which has been described by Fletcher and Hopkins[3].

The contraction of water rigor is slow. In the sartorius it often does not begin for four or five minutes and takes several hours for completion. Both the preliminary period, in which there is no contraction, and the time necessary for the contraction to complete itself, are longer in larger muscles. It is not to be expected, therefore, that much lactic acid would be found in larger muscle in less than an hour.

In carrying out the experiments I have followed the routine described by Fletcher and Hopkins. Pairs of frogs' legs were skinned and kept for varying periods in distilled water. The muscles were then crushed under ice-cold alcohol and extracted, and the extracts were tested by the thiophene test. In every case the reaction of the extract from the muscles in water rigor was compared with that of the extract from a control set of muscles which had been kept for the same length of time in Ringer's fluid or in a moist chamber. In this way

[1] du Bois Reymond. *Muskel- und Nervenphysik*, II. p. 17. Leipzig, 1877.

[2] The Ringer's fluid used in all the experiments described in this article, had the following formula;—NaCl, 65 grams; KCl, 2 grams; $CaCl_2$, 2·5 grams; $NaHCO_3$, 1·5 grams; distilled water, 10 litres.

[3] Fletcher and Hopkins. This *Journal*, XXXV. 308. 1907.

the amount of lactic acid was roughly determined in muscles which had been in distilled water for 1, 1½, 2, and 5 hours respectively. In all cases the extracts from the muscles in water rigor gave more strongly positive tests than the controls, and the change was shown to be progressive, that is, the greatest difference between the muscle in water rigor and its control was found in the case where both had been in their respective fluids for 5 hours, the next greatest difference was in the 2 hour experiment, and so on. There is no doubt, therefore, that the contraction of water rigor is accompanied by a slow lactic acid production.

Acid formation in rigor caused by hypertonic solutions.

It is known that when muscles are placed in hypertonic solutions they shrink and shorten in the course of a few minutes. It seemed to me of interest to determine whether this shortening is accompanied by the formation of acid. Accordingly, I placed gastrocnemius and sartorius muscles in 50% glycerine, in 50% cane sugar, in 10% NaCl, and found that the muscles gave an acid reaction to litmus paper after five minutes stay in any of these fluids, and that in ten minutes the reaction became intense.

Relation of increase of weight to decrease of length in muscles placed in distilled water.

Fletcher[1] has shown that the increase in weight of the sartorius which occurs when it is immersed in ·2% NaCl soon ceases. In about half-an-hour, it begins to lose weight, giving up the water previously absorbed.

I find the same phenomena, but to a more marked extent, when the muscles are placed in distilled water instead of in dilute salt solution. The increase of weight of the sartorius is over in about twenty minutes, and a loss of weight sets in which continues for several hours.

It is known that the shortening of the muscle in water rigor continues for a much longer period than that just given for the absorption of water, so that the two phenomena can only be indirectly connected with one another. The absence of direct causal connection is also shown by the different effects of variations in temperature.

Six sartorii as nearly as possible of the same size were arranged to record their changes in length on a kymograph and immersed for half-an-hour in distilled water at 0°, 5°, 10°, 15°, 20°, and 25° respectively.

[1] Fletcher. This *Journal*, xix. p. 423. 1904.

At the end of the half hour the first had shortened 2·1 % of its original length; the second, 5·7 %; the third, 8 %; the fourth, 8·3 %; the fifth, 10·6 %; and the sixth, 19·3 %.

On the other hand, the rate at which muscles immersed in distilled water gain in weight is little if at all dependent on temperature. I have carried out a number of experiments in which fellow sartorii were immersed in distilled water at various temperatures and weighed at intervals. The results of a typical experiment are given in Table I. They show quite conclusively that the rate at which muscles immersed in distilled water gain in weight is very much less dependent on temperature than the rate of shortening under similar circumstances.

TABLE I.

Immersion of sartorius muscle in distilled water at various temperatures.

		Weight 1		Weight 2
Fresh		0·09 gm.		0·09 gm.
After 5 minutes in distilled water	At 0°	0·112	At 15°	0·118
After 10 minutes in distilled water		0·130		0·140
After 15 minutes in distilled water		0·144		0·148
After 20 minutes in distilled water		0·155		0·149

Thus at 15° the swelling is complete at the end of twenty minutes, while the contraction has only well started, and goes on for hours during which the muscle is losing water. At 0° the muscle may lengthen during the first ten minutes of its immersion, so that the swelling is nearly complete before the shortening begins at all. The time relations between the shortening and the increase in weight of muscle immersed in distilled water are graphically shown in Figs. 1 and 2. The exact form of the curves was not very carefully determined, for it varies greatly in different muscles under the same conditions.

The effect of Ringer's fluid on the disappearance of rigor and of muscle acidity.

I have not yet found[1] a stage of the water rigor contraction in which it is not practically completely reversible. In one case a sartorius was arranged to record its changes in length, weighted with 0·5 gram, and immersed for two hours in distilled water. During this period it shortened 28 % of its original length. Ringer's fluid was then substituted for the distilled water, and in the course of five hours more the muscle relaxed to its original length. The room temperature during this experiment varied between 14° and 15° C. The muscle had, of course, irretrievably lost its irritability long before the end of the first two hours.

[1] I have still some observation to make on the later stages.

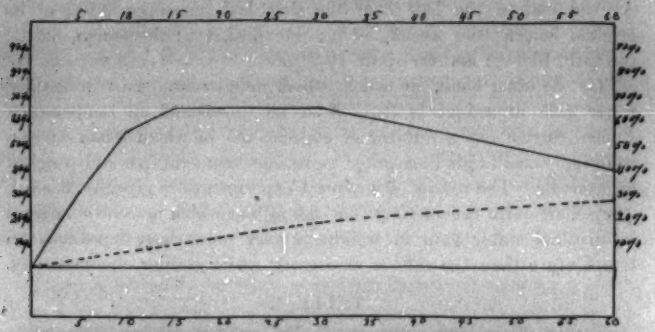

Fig. 1. Diagram to illustrate the time relations between the changes in weight and the changes in length which take place in a sartorius immersed in distilled water at 15° C. The continuous line represents changes in weight, and the dotted line, changes in length. The figures along the abscissa give the time in minutes; those along the ordinate give the percentages of increase in weight and decrease in length respectively.

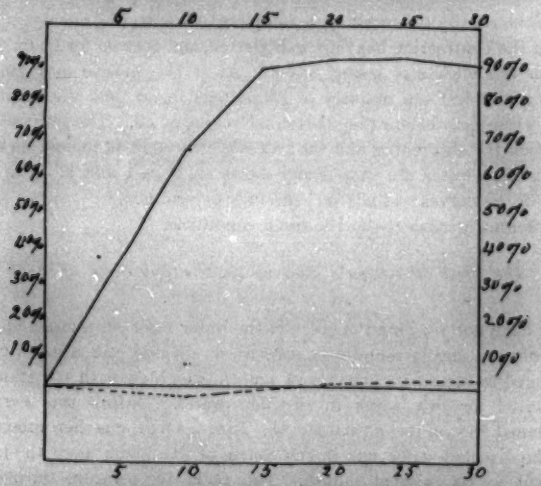

Fig. 2. Diagram to illustrate the time relations between the changes in weight and the changes in length which take place in a sartorius immersed in distilled water at 0° C. The same conventions are used as in the preceding diagram.

Half-an-hour's immersion in distilled water is quite sufficient to destroy permanently the irritability of a frog's sartorius.

To determine the question whether the acid reaction produced by distilled water is removed by subsequent immersion in Ringer's solution, I have carried out experiments, of which the following is a typical example. A sartorius, which will be called sartorius 1, was arranged to record its changes in length and immersed first for twenty minutes in distilled water at 16° and then for three hours in Ringer's solution at 16°. After the twenty minutes in distilled water it had shortened 14·6 %, and during the three hours in Ringer's solution it relaxed to its original length. Toward the end of its immersion in Ringer's solution sartorius 2 from the opposite leg of the same frog was immersed for twenty minutes in distilled water at 16° and shortened 18·7 %. The reaction of the two muscles to blue litmus was then taken. Sartorius 1 was, so far as could be determined from the rough litmus test, as neutral as a fresh muscle; sartorius 2 was distinctly acid.

In other cases the muscles were left for longer periods in the distilled water and Ringer's fluid; sometimes only one muscle was used, and its reaction after a period in distilled water compared with its reaction after a subsequent period in Ringer's fluid. Experiments were also tried to exclude the possibility that muscle becomes acid and then loses its acid reaction during a long period of immersion in distilled water. This is not the case; a frog's sartorius remains strongly acid in distilled water for periods extending over more than four hours.

The results of all the experiments were quite consistent with each other, and leave no doubt of the fact that the acid reaction produced in muscle by distilled water is removed by subsequent immersion in Ringer's fluid. Whether the removal of the acid is simply by diffusion, or partly in consequence of the fluid containing $NaHCO_3$, I have not determined.

Remarks and Summary.

It was shown by Overton[1] that the sartorius in ·4 % NaCl swells up and becomes white and opaque without losing its irritability. Fletcher[2] found that in ·2 % NaCl, the swelling decreases after about half-an-hour, while it is known that the shortening of rigor is a much more protracted process. It was shown by myself[3] that in ·3 % NaCl, the muscle may swell considerably without shortening. Thus in hypotonic

[1] Overton. *Pflüger's Arch.* xcii. 144. 1908.
[2] Fletcher. *Op. cit. supra.*
[3] Meigs. *Amer. Journ. Physiol.* xxii. p. 498. 1908.

salt solutions the absorption of water, and the rigor proper are separable phenomena. This is also the case when the muscle is placed in distilled water. The swelling begins to decrease in about 20 mins., whilst the shortening continues for some hours. Moreover the rapidity of shortening is highly dependent upon temperature, whilst the swelling is little if at all dependent on it.

The distinction here drawn between the early rapid temporary swelling of the muscle in distilled water, and the slow continuous shortening which supervenes upon it, serves, it may be suggested, to reconcile du Bois Reymond's[1] observations of the acidity developed in water rigor, with those of Biedermann and of Fletcher, which indicate, on the other hand, an absence of conspicuous chemical change. Both Biedermann[2] who showed that immersion in water produces little if any change in the electrical potential of the muscle, and Fletcher[3] who found no increase in the survival CO_2 discharge of the immersed muscle, were concerned with the early stages of water-swelling before the development of acidity, with the shortening of the muscle, had advanced far towards final rigor.

In the cases which have been investigated, it has been found that the shortening of the muscle in rigor is accompanied by the formation of acid. I have shown above that this is also the case in the rigor caused by glycerine, and by hypertonic solution of cane sugar, and of NaCl, and that in water rigor, acidity is partly at any rate due to lactic acid.

There is a considerable body of evidence that lactic acid is the cause of the shortening of the muscle in rigor, and is not simply an accompaniment of it. Thus Fletcher[4] found that there was no rigor in the frog's sartorius in an atmosphere of oxygen, and Fletcher and Hopkins[5] that in oxygen, little or no lactic acid is accumulated. It has been shown in the preceding pages that if a muscle in water rigor is placed in Ringer's solution, it loses acid and at the same time lengthens. The fact seems to me to strengthen the view that the shortening of the muscle in rigor is caused by the acid.

[1] du Bois Reymond. *loc. cit.*
[2] Biedermann. *Elektrophysiologie*, p. 305. Jena, 1895.
[3] Fletcher. This *Journal*, XXIII. p. 87. 1898—99.
[4] Fletcher. This *Journal*, XXVIII. p. 478. 1902.
[5] Fletcher and Hopkins. This *Journal*, XXXV. p. 283. 1907.

SOME NEW FORMS OF APPARATUS FOR THE ANALYSIS OF THE GASES OF THE BLOOD BY THE CHEMICAL METHOD.

By T. G. BRODIE, M.D., F.R.S.

(From the Physiological Laboratories of the London School of Medicine for Women and the Brown Institution, University of London.)

In the course of investigations in which I have used the Barcroft-Haldane apparatus for the estimation of the gases of the blood I have introduced several modifications which, I think, simplify the use of the apparatus and increase the rapidity with which the analyses can be made. As, in most experiments, several analyses have to be made, and as, too, it is very essential that they should be carried out rapidly I have gradually introduced a number of time-saving devices for the collecting and measuring of the various volumes of blood and liquid used in the method. These are described in the following sections.

The modified blood gas apparatus. Each pair of manometer tubes is mounted on a small wooden base (see Fig. 1) in such a way that the tubes can be easily removed for cleaning purposes. The two tubes are united at the bottom and at the lowest part of the bend a wider tube is fused in. To this a piece of rubber tubing is attached. This tubing can be compressed by a screw clamp, *E*, and thus the level of the fluid in the manometer tubes adjusted. The most important modification in the apparatus is in the form of the bottle. Since each apparatus is small it is now possible to have the bottle rigidly attached instead of being fitted in position by rubber tubing. In this way the volume of the air space in the apparatus is always quite constant. The contents of the bottle can be vigorously shaken by shaking the whole apparatus without running any risk of endangering the manometer reading. I have discarded the little glass cup into which the ferricyanide solution was placed because this was difficult to clean and was frequently broken.

The following arrangement takes its place. The stopper, *F* (Fig. 2), of the bottle is made of good size and the manometer tube is attached to it at one side. Fused into the side of the stopper is a small bent glass tube, *G*, which opens through the side. The neck of the bottle has a small recess, so that when the bottle is placed on the stopper the lower end of the tube, *G*, is closed in all positions of the bottle except when the recessed part faces the lower end of the internal tube. By removing the small stopper, *J* (Fig. 2), the ferricyanide solution can be placed in the tube *G*, when the bottle is in position. The proximal manometer tube has a single mark, *L*, upon it; the other tube is graduated in

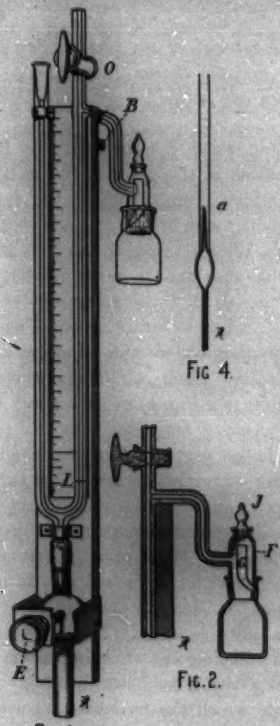

FIG. 1. FIG. 4. FIG. 2. FIG. 3.

millimetres, or the tubes are mounted on a glass mirror graduated in millimetres. The capacity of the bottle and of the tube down to the mark *L* is determined by filling with mercury and then weighing the mercury.

I at first experienced considerable difficulty in getting the manometer tubes sufficiently clean to give the readings accurately. This difficulty was at last overcome by employing a dilute solution of bile salt wherewith to fill the manometer and it then does not matter if the tubes are not perfectly clean. The solution I use consists of a 0·5 % solution of bile salt in which a little toluol is dissolved. Sodium chloride is then added until the specific gravity of the solution is 1,034. If desired a little pigment may be added to the solution, but it is quite easy to read the manometer without this.

When attaching the bottle to the manometer tubes for an analysis great care must be observed that the bottle is pushed well home on the stopper, otherwise in turning it during the analysis an alteration may occur in the manometer zero. It is best to use a very small amount of thin vaseline on the stopper as a lubricant, and each apparatus should be tested while empty to prove that rotation of the bottle does not alter the manometer zero.

On the back of each manometer there is a bent metal support, *B*, by means of which the apparatus can be suspended from the side of the cooling bath.

The blood sampling pipette. This pipette is illustrated at *K*, Fig. 3. It consists of a tube of about 6 c.c. capacity. Its lower end is provided with a tap terminating in a long fine nozzle. The upper end has a constriction and here there is a fine mark, *a*, encircling the tube. The capacity of the tube down to the mark *a* is accurately determined. When the tube is to be used for collecting a blood sample it is first held vertically in a clamp and a Hg reservoir attached to the lower end by rubber tubing. The upper end is connected by a short piece of rubber tubing with a fine glass tube bent so as to project downwards. The tube is now nearly filled with Hg and then a solution of hirudin (1 %) in Na Oxalate (4 %) is drawn into the tube. When about 1 c.c. has entered the Hg reservoir is again raised and the hirudin solution run out until the Hg stands exactly at the mark *a*. This leaves a known quantity of the anticoagulating solution in the tube. It is now ready for receiving the sample of blood. The clamp on the vein or artery is first opened and the cannula and short length of rubber tubing is filled with blood from the vessel, enough being allowed to escape to wash out the

tube thoroughly. The sampling tube is then attached to the cannula by rubber tubing, and by opening the tap mercury is run out slowly into a small measure until a sufficient amount of blood (about 4 c.c.) has been drawn into the tube. The vessel is then clamped and the tube removed. A finger is at once placed over the open orifice, *b*, the tube inverted and by a sharp downward swinging movement Hg is driven into the capillary orifice to seal off that end. The tube is then turned upwards and downwards a few times to mix blood and anti-coagulating fluid thoroughly. It is then placed upper end downwards in a vessel containing Hg which seals up the open end until the blood is required for the analysis.

The amount of Hg collected gives the volume of blood taken into the tube. The capacity of the tube down to the mark, *a*, being known the amount of dilution of the blood is known and allowance for this dilution can be made when calculating the blood gases.

The automatic pipette for delivering the blood into the analysis bottle. This pipette is shown in Fig. 3. It consists of a long tube, *A*, 43 cms. in length, the upper half of which is graduated for 1 c.c. in 1/100 c.c. The upper end is closed in by a small cover, *F*, to catch any mercury which escapes when filling. On this tube there is a tap, *B*. A second vertical tube, *C*, is provided above with a receiver to hold mercury. It also is provided with a tap, *D*, and a little lower turns horizontally and shortly after the first tube joins it as in the figure. A little further there is a third tap, *E*, and finally the end is turned up and has a short length of thick walled rubber tubing on it. Over this again is a glass cup, *H*. It is used in the following way. The whole of the tubing and cups being filled with mercury the sampling tube containing the blood is attached so that the fine nozzle of the tap end of the tube fits into the rubber tubing under the mercury until the two glass ends lie in apposition (see Fig. 3). To the upper end of the sampling tube a bent glass tube of capillary bore is attached, and on opening taps *D* and *E* mercury is run in and the mercury seal and a little blood discharged from the sampling tube, enough blood being run out to wash out this fine tube thoroughly. The tap *D* is then turned. The bottle of the analysing apparatus is now held so that the orifice of the fine tube delivering the blood touches the bottom of the bottle and by opening tap *B* exactly 1 c.c. of blood is displaced into the bottle.

Before receiving the blood 2 c.c. of dilute NH_3 solution (3 c.c. NH_3 ·880 in 1 l. distilled water) is run into the bottle. This is delivered by means of a somewhat similar pipette to that just described. In placing

this NH_3 solution in the bottle great care must be taken to protect it. It should be done in front of an open window and a glass plate of good size should be placed in front so that the expired air of the operator cannot by any possibility be blown into the bottle. The two bottles are only filled immediately before being required. The blood is then at once run in and the bottles quickly attached to their proper manometers.

When attaching the bottle to the manometer the tap *O* at the top of the manometer tube must be open and the little stopper *J* removed. The bottle is then ground well home and left in such a position that the lower end of tube *G* is closed. The proper quantity of ferricyanide solution is then put in by a choked pipette (Fig. 4) with a very fine capillary orifice at *a*.

The volume of fluid this delivers is previously carefully estimated by weighing. The fluid is drawn up and discharged by a small syringe, not by the mouth, so as to prevent any possible inclusion of expired air in the apparatus. The stopper *J* is then inserted and the apparatus placed in the cold water bath.

The water bath. This consists of a long copper trough 93 cms. long, 9·5 cms. wide and 11·5 cms. deep. Along the back and lower angle runs a metal tube with a large number of fine holes bored through it. One end of this tube is closed and the other is attached to the water supply and the water is then driven into the bath through the fine holes. The bath is thus filled, excess of water escaping through a side tube at the top of the bath. In this way there is a continuous stream of water coming in from the main water supply by a number of fine jets. These jets are directed obliquely upwards so that they impinge on the bottoms of the bottles on the manometers and in this way there is a continuous stirring of the water of the bath. The temperature too very rarely varies, and if it does it is only to a very small extent.

The bath is long enough to receive a row of 20 manometers. It stands upon a wooden support with a longitudinal strip at the base to steady the lower ends of the manometers. In most experiments eight samples of blood, 4 venous and 4 arterial have been analysed. Each is done in duplicate, thus 16 manometers are necessary. A seventeenth is also used to act as a temperature control and to estimate the amount of CO_2 in the ammonia solution. For this purpose the experiment is carried out in this apparatus just in the same way as in all the others with the exception that no blood is placed in it. As soon as the taps of two manometers are closed to commence the observation, that of the temperature control is also closed and read. Each time a fresh reading

of a manometer is made a temperature reading is also taken so that the necessary correction, if any, may be made. The analysis is carried out as described in the paper by Barcroft and Haldane[1]. After the temperature has become constant the tap *O* is turned, the blood is laked and allowed to stand for 1 min. The bottle is then rotated and the ferricyanide allowed to run in. It is then well shaken. I find that shaking for 1 min. is usually sufficient to disengage all the oxygen. The apparatus is then cooled and read. If the manometers have been sorted out into pairs of nearly equal capacity, the readings for the same blood ought to agree. This is a great convenience in determining if all the O_2 has been disengaged. After the readings have been taken the taps, *O*, are opened, the manometers taken out, the bottles wiped and the stopper *J* removed. The saturated tartaric acid solution is then run in from the pipette as before and the whole operation repeated for the CO_2 determination. With two workers it is possible to carry out an experiment including the 16 blood analyses in five hours. It is best to get the blood into the apparatus as quickly as possible. Usually with two workers this is done within 2 or 3 minutes after each sample has been taken.

The apparatus described in this paper may be obtained from Messrs F. E. Becker & Co., Hatton Wall, Hatton Gardens, London, E.C.

[1] This *Journal*, XXVIII. p. 282. 1902.

ON THE RELATION BETWEEN THE PHYSICAL, CHEMICAL AND ELECTRICAL PROPERTIES OF THE NERVES. Part II. THE TISSUES COMPOSING A NERVE TRUNK.

By F. O'B. ELLISON.

(From the Physiological Laboratory of St Mary's Hospital Medical School.)

In uniformity with the later experiments described by Alcock and Lynch in Part I[1] the estimations were carried out on the medullated (Median) and non-medullated (Splenic) nerves of the horse.

Technique. The chief difficulties lay in the practical impossibility of obtaining sections which would show the exact disposition of tissues during life. Formol in various strengths, Mann's fluid, acetic-bichromate mixtures and Flemming's fluid, followed by paraffin embedding, all caused abundant evidence of shrinkage, even though the tissues were hardened in a carefully graduated series of alcohols. Paraffin embedding was accordingly discarded in favour of celloidin embedding. In the latter medium, the shrinkage, though not entirely obviated, was slight, and such as was present manifested itself in such a way that allowance could readily be made for it. Sections cut in celloidin of a thickness of 10 μ were found convenient for staining and subsequent observation.

In the present paper, forming No. II of the series, an estimation is made of the amount of the various tissues composing a nerve trunk.

The technique finally adopted was as follows: the tissue was fixed for 4—10 hours in Flemming's fluid; washed in running water for 24 hours; allowed to remain for 12 hours in alcohol 30, 40, 50, 60, 70, 80, 90, 95 %; and then for 24 hours in absolute alcohol; placed for 24 hours in 3 % celloidin solution (Schering's chips dissolved in equal parts absolute alcohol and anhydrous ether), and for 24 hours in 12 % celloidin. The celloidin block was hardened in 70 % alcohol.

The sections were stained for one minute with a saturated aqueous solution of acid fuchsin, washed with methylated spirit, cleared with a mixture of equal parts cedar oil, clove oil and phenol crystals, and finally

mounted in balsam. This was found to give the best differentiation between connective tissue, medullary sheaths and axis cylinders: the connective tissue being stained red, the medullary sheaths black, and the axis cylinders mostly unstained.

In order to determine the amount of shrinkage brought about by the processes of fixation and embedding, a second series of sections was prepared from fresh nerves. These were frozen and cut without embedding, being merely covered with a drop of 1 % NaCl solution [2]. The sections were about 25 μ thick. They were thawed in 1 % NaCl solution and mounted and examined in the same medium. From these sections drawings were made on thin cartridge paper with high and low powers of the microscope, the low power magnification being about 25, the high power 500—1500.

Photo-micrographs were also prepared with magnifying powers of 50 and 1000 using green monochromatic light from the apparatus described by Alcock [3]. The negatives so obtained were printed on a collodion paper, as being less hygroscopic than gelatine.

The drawings and photographs with the low powers were cut up with sharp scissors along the lines separating the perineurium from the bundles of fibres contained inside. These two sets of pieces, the round bundles of nerve fibres, and the irregular portions representing the connective tissue between the bundles, were then placed in separate weighed bottles, dried *in vacuo* in a desiccator over sulphuric acid for 24 hours and then weighed. In cutting out the pieces, any portions representing holes in the section, due to shrinkage, were discarded.

In the case of the high power drawings of medullated nerve, three divisions were made, the drawings being cut up into axis cylinder, medullary sheath, and connective tissue, in this case endoneurium. Any non-medullated fibres present in the medullated nerve was cut out and placed in the axis cylinder bottle. These three portions also were dried *in vacuo* and weighed.

It was only possible to obtain photo-micrographs from fixed and stained preparations, but comparison between photo-micrographs and drawings of fixed and stained sections showed a close agreement between the photograph and the drawing by hand.

Assuming that the paper on which the drawings or photographs were made was of uniform thickness and density, the sectional area of each tissue will be proportional to the weight of the area occupied by that tissue in the drawings. Assuming also that the sides of the nerves are parallel and the constituents of the nerves all of the same density, the actual amount of each tissue present will vary as the sectional area

of the same, and hence will be proportional to the weight of the pieces of paper cut out of the drawing.

Two chief difficulties were met with.

(*a*) In the case of fixed and hardened tissue a certain amount of shrinkage was unavoidable.

(*b*) In the case of fresh tissues stretching of the sections occurred. This took place chiefly in the epineurium, the highly elastic connective tissue between the bundles of fibres allowing the latter to separate from one another to a variable distance. This was most marked when the cover glass was placed in position, especially if the amount of fluid under the cover glass was not very great.

In order to minimise the error due to preparation, the mean in the final result was taken between the figures given by fresh and fixed preparations.

The figures obtained were as follows:

TABLE I.

Percentage of constituents of the median and splenic nerves of the horse.

		Per cent. of whole nerve (low power)		Bundles of fibres per cent. (high power)		
Observation		Connective tissue	Bundles of fibres	Endo-neurium	Medullary sheath	Axis cylinder or nerve-fibre
1 *a*	Median, fixed (photograph)	38	62	—	—	—
2	Median, fresh (drawing)	55	45	—	—	—
3 *a*	Median, fresh (drawing)	46	54	—	—	—
1 *b*	Median, fixed (photograph)	—	—	41	42	17
1 *c*	Median, fixed (drawing)	—	—	42	40	18
3 *b*	Median, fresh (drawing)	—	—	28	59	13
3 *c*	Splenic, fresh (drawing)	28	72	—	—	—
3 *d*	Splenic, fresh (drawing)	32	68	—	—	—
1 *d*	Splenic, fixed (drawing)	—	—	46	—	54
4	Splenic, fixed (drawing)	—	—	42	—	58

The different observations made on the nerves of the same animal are shown by lettering, thus:—1 *a*, 1 *b*, 1 *c*.

TABLE II.

Percentage composition of whole nerve deduced from Table I.

Observation		Connective tissue	Medullary sheath	Axis cylinder
1	Median, fixed	63 %	26 %	11 %
3	Median, fresh	61	32	7
5*	Median, fixed	66	23	11
6†	Splenic	61	—	39

* This is taken from a former estimation by the photographic method by Alcock.

† Average of two mixed estimations, the low power drawings from fresh, the high power from fixed material, in the Table I enumerated 1 *d*, 3 *c* and *d* and 4.

Controls.

From the figures given in Table I allowance can be made for four variable factors.

(1) Variation in different drawings of the same nerve from the same animal (3 *c* and 3 *d*).

(2) Variation in the same nerve from different animals (2 and 3 *a*).

(3) Variation between drawings and photographs of the same section of the same nerve from the same animal (1 *b* and 1 *c*).

(4) Differences between fresh and fixed material (1 *c* and 3 *b*, 1 *a* and 2).

As a control of the histological method generally the following experiment was carried out on the amount of connective tissue.

Portions of the median nerve of a horse (34) was taken and fixed in strong Flemming's fluid without being previously cleaned from epineurium, a second similar portion was carefully cleaned as far as possible by Mr Lynch in the same manner and to the same extent as employed by him and Dr Alcock in their experiments on the chemical composition of nerve. This portion was also fixed in Flemming's fluid, and sections of both cut of a thickness of 20 μ. Outline drawings of both were then prepared, and the same amount of peripheral connective tissue marked off on the drawing of the nerve which was not cleaned, as appeared to have been removed from that which was cleaned. This was then cut off from the drawing, and both parts dried and weighed and the ratio determined from the relative weights as above described.

A second piece of the nerve was taken, weighed before being cleaned and then cleaned in the same way, and again weighed. The difference of course was equal to the amount of tissue cleaned off, *minus* a little loss due to evaporation. The percentages of connective tissue (epineurium) removed, as estimated by the two methods, were then compared.

Histological method.

Weight of drawing of section of whole nerve	0·552.
" " " cleaned "	0·429.
Difference, connective tissue removed	0·123.
= 22·3% of whole nerve.	

Gravimetric and dissection method.

Weight of nerve before cleaning 3·710.
" " after " 3·025.
Difference = connective tissue = 0·685 = 18·4% of whole nerve.

This experiment shows the accuracy of the method at its least accurate point.

Conclusions.

The relative amount of the different constituents of a medullated and of a non-medullated nerve of the horse, were determined by drawing and photographing transverse sections of the nerves, cutting out the parts of the pictures occupied by each constituent, and drying and weighing each part.

The average percentage composition was as follows:

Medullated nerve (Median)—

Connective tissue	63 %
Medullary sheath	28 %
Axis cylinder	9 %
	100

Non-medullated nerve (Splenic)—

Connective tissue	61 %
Nerve fibre	39 %
	100

The percentage of epineurium and perineurium in the median nerve was also determined by weighing the nerve before and after dissecting off the epineurium; the results agreed with that obtained by the previous method.

In the microscopical sections no confirmation was found of Tuckett's[4] observations as to the presence of a sheath to the non-medullated nerve fibres.

REFERENCES.

(1) Alcock and Lynch. This Journal, xxxvi. p. 93. 1907.
(2) Ibid. p. 96.
(3) Alcock. Science Progress, vi. Oct. 1907.
(4) Tuckett. This Journal, xix. p. 267. 1896.

ON THE RELATION BETWEEN THE PHYSICAL, CHEMICAL, AND ELECTRICAL PROPERTIES OF THE NERVES[1]. Part III. TOTAL ASH, SULPHATES, PHOSPHATES.

By N. H. ALCOCK, M.D., D.Sc., AND G. ROCHE LYNCH.

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Introduction. In the present paper are contained the results of the analysis of the ash of the tissues which compose a nerve trunk, together with the considerations arising out of the determinations of the total sulphur and phosphorus found in the same tissues and the relations which these figures might bear to the subject of these researches. Special attention has been paid to the determination of the sulphates and phosphates in the ash, as these substances illustrate very clearly what has been known for some time and often forgotten, that the ash of any tissue is not a measure of the electrolytes originally present, nor can the conductivity of a solution made up by dissolving the ash in a bulk of water equal to the tissue, throw any light on the original distribution or amounts of the ions, or on the structure of the tissues.

The analyses were made on the nerves of the horse. From each animal can be obtained 10 to 20 grams of median nerve, 3 to 5 grams of splenic, and about the same amount of connective tissue. As from 70 to 80 % of the tissues consists of water and the total ash is not more than 1 %, it is evident that the collection of sufficient material is a matter of some little time even if the analyses are carried out on small quantities of the substance. Very great accuracy is not to be expected

[1] The expenses of this research have been defrayed in part by grants from the Royal Society.

under such conditions, and, in view of the individual differences of different animals, such accuracy if possible would be meaningless. For the present purpose the figures actually obtained amply suffice, and it will be seen that the validity of the conclusions does not depend on the correctness of the last decimal place.

A nerve trunk contains a considerable quantity of connective tissue, so that in order to ascertain the distribution of the various electrolytes in a nerve fibre as well as their total amount it is necessary to make analyses of medullated nerves, non-medullated nerves, and connective tissue.

Knowing the amount of any given electrolyte is these three tissues and knowing also the relative amount of each tissue in a nerve trunk (as Ellison has determined in Part II of this series) it should be possible to make at least a probable estimate of the manner in which each electrolyte is distributed in each part of the nerve.

General methods. The three tissues examined (1) the median nerve, (2) the splenic nerve, (3) axillary connective tissue, were removed from the animal as soon as it had been killed. In the laboratory the wet weight of each was determined, and this weight serves as the reference point for all the analyses.

Before weighing the nerves it is necessary, as has been previously explained, to clean off the epineurium, as this amounts to about $\frac{1}{8}$ of the total weight. It is an easy matter to dissect off this tissue and leave the nerve fibres clean and free from adherent blood. Due care was taken to prevent as far as possible any drying of the nerve during the process.

In the case of connective tissue the matter is not so simple. If the animal is not too fat and has been bled after being shot, it is easy to take 4—5 grams of white and clean connective tissue from the axilla which will contain a comparatively small amount of adipose tissue and very little blood. But under the reverse conditions it is found that the connective tissue may be so loaded with fat and so vascular as to make a distinction impossible between connective and adipose tissue, and in such an animal the estimation must be omitted.

When the wet weight of the nerve or tissue had been determined the material was in most of the experiments dried at room temperature in a vacuum over pure H_2SO_4. In the later series of experiments the nerves were dried over $CaCl_2$, the analyses showed no difference, outside the experimental error, between the two methods of desiccation. The dried tissue was then taken for analysis.

Ash of nerve.

In the first three series of experiments the ash of the nerve was obtained as a preliminary step. Medullated nerve is a tissue which oxidises with difficulty, and it is necessary either to use a bright red heat for 2—3 hours or some oxidising agent. The best result was obtained with ammonium nitrate. With this it is not necessary to go above a dull red heat but there is apt to be some loss from spurting, and, probably, some loss of chlorides. Splenic nerves and connective tissue burn much more readily, but were put through the same process in order to obtain comparable results.

The details of the experiments are given in the tables under Series I, II and III, later, the mean values only are quoted here. They are expressed as percentages of the original wet weight of the nerve or tissue.

TABLE I.

	Ash with NH_4NO_3		Ash by heat alone	
Tissue	Ash % of wet wt.	No. of horses used	Ash % of wet wt.	No. of horses used
Median Nerve	·985	12	·995	6
Splenic Nerve	·914	2	·635	11
Connective Tissue	·549	3	·343	10

It will be seen from Table I that the median nerve has rather more ash than the splenic and connective tissue less than either.

It was found that as regards the amounts of sulphate and phosphate present in the ash the same result was obtained by the simpler method of using heat alone, and as this was more convenient it was adopted for the later determinations. The "total" ash obtained in this way is naturally of a different composition and weight from the ash obtained by the former method. The difference is greatest in the case of the splenic nerve and connective tissue.

Determination of sulphates and phosphates in the ash.

A. Sulphates. The platinum crucible containing the ash was heated with distilled water containing about 1 % HCl in a Jena glass beaker on a water bath for 2—3 hours. The platinum crucible was then washed, heated and again weighed, to make sure that all the ash had been extracted. The extract was treated with excess of pure $BaCl_2$

and boiled in the water bath for about 8 to 12 hours. The precipitate of $BaSO_4$, which as a rule only made its appearance after 2—3 hours boiling, was then filtered off through an ashless baryta filter paper and estimated gravimetrically in the usual way. The filtrate was reserved for the estimation of phosphates.

As in the case of the ash, the analysis of the median nerve presented considerable difficulties. Even when 23 grams of fresh nerve were taken from three horses the $BaSO_4$ precipitate weighed but ·0066 gram and this had to be estimated in a fluid containing approximately 60 times its weight of phosphates. It was necessary to make a large number of preliminary experiments with mixtures of known amounts of H_2SO_4 and sodium phosphate before any reliance could be placed on the method of analysis, and even under the most favourable circumstances much care is needed when dealing with such small weights of precipitate. The accuracy depends chiefly on a sufficient length of time spent in boiling. When this time exceeds 8 hours the error from imperfect precipitation is not more than 1% at the most. Less than 4 hours boiling introduces a considerable error from this cause. We did not observe under the conditions used any appreciative error due to precipitate of other substance than $BaSO_4$.

The splenic nerve and connective tissue ashes present no special difficulty, apart from the small amount of substance taken.

B. Phosphates. The filtrate from the above experiment was then boiled with H_2SO_4 to remove the excess of $BaCl_2$ and filtered. The filtrate was then made alkaline with ammonia. In some cases the beaker was left to stand over night so as to see if any precipitate of Mg, Ca, or other phosphates would occur. No more than a faint cloud of precipitate of unweighable quantity ever appeared. Magnesia mixture was added in excess in the usual way and after 24—48 hours the precipitate collected on a platinum Gooch crucible and estimated in the usual way.

SERIES I. *Median Nerve.*

Exp.	Method of ash	Ash % of wet wt.	SO_4 %	PO_4 %
1	NH_4NO_3	1·008	—	·770
2	,,	·924	·0117	—
3	Heat	·994	·00934	·770
4	,,	·995	·0116	·774
5	Coal	—	·0116	·759
Mean	—	·984	·0110	·768
6	Heat (ether)	·887	·0182	·607

In Exps. 1 and 2 the ash was obtained with the help of NH_4NO_3, in Exps. 3 and 4 by heat alone.

In Exp. 5 the nerve was roasted at a temperature that never rose as far as visible redness. The coal thus obtained was extracted repeatedly with dilute HCl, the extracts mixed and the sulphates and phosphates determined as before. The figures by all the methods agree within the limits of error.

In one experiment, No. 6, the conditions were varied and the result was slightly different. The dry nerves were extracted in a Soxhlet apparatus with pure dry ether (which had been dried and distilled over sodium), and the ether extract and extracted nerve ashed separately. The ash of the ether extract weighed only ·0051 gram, contained no sulphate that could be detected and appeared to consist almost entirely of phosphates. Although the total ash was less than usual, the proportion of sulphate was higher and the experiment suggested an explanation (to be considered later) of the very small percentage of sulphate otherwise obtained.

SERIES II. *Splenic Nerve.*

Exp.	Method of ash	Ash % of wet wt.	SO_4 %	PO_4 %
7	NH_4NO_3	·920	·0658	—
8	,,	·914		
9	Heat	·681	·0769	·352
10	,,	·619	—	·347
11	Heat (ether)	·605	·0514	(·319)
Mean	NH_4NO_3	·917	·0644	·329
	Heat	·635		(Omitting Exp. 11)

Connective tissue is in some respects much more variable than either medullated or non-medullated nerves and a series of preliminary experiments were undertaken to determine how far the analyses were comparable with the analyses of the nerve fibres.

Percentage of water. The tissue was dried at room temperature in vacuo over pure H_2SO_4 (see Part I).

TABLE II.

Exp.	H_2O %	Exp	H_2O %
12	81·5	17	70·5
13	78·6	18	79·0
14	86·6	19	86·6
15	87·5	20	76·5
16	79·0	Mean	80·7

The mean is obviously not of much value, and if more than an approximation is required each portion of tissue must be analysed separately.

As the percentage of fat increases the percentage of water falls off.

TABLE III.

Exp.	H_2O %	Notes
21	60·3	—
22	58·7	—
23	45·7	Visible fat.
24	42·2	,, ,,
25	24·6	Indistinguishable from adipose tissue.

The wet weight of the tissue, which has been taken as to reference point hitherto, is therefore misleading in such cases. Some experiments were undertaken to see what results would be obtained if the value "wet weight less fat removed by ether" were used as a basis for the calculation. The method adopted was the same as in the case of the nerves, the fat being extracted from the dry tissue with pure dry ether.

TABLE IV.

Exp.	A original wet wt.	H_2O %	B wet wt. *less* fat extracted	Ash % of A	Ash % of B	SO_4 % of A	SO_4 % of B
26	11·078	77·8	10·212	·318	·335	·0552	·0726
27	12·167	44·2	7·391	·180	·291		
28	19·476	24·6	6·691	·067	·194	·0211	·0614

Purposely extreme cases were taken and it is evident that the main variant is the amount of fat originally present, as when the ash and SO_4 are calculated in percentages of **B** the results are more concordant than when calculated on **A**. But the difficulties that exist in comparing the weight **B** with the conditions in nerve fibres led us to abandon this method of calculation, and to minimise the error due to the varying amount of fat by taking for analysis only those samples of connective tissue that had a high and fairly constant percentage of water.

SERIES III. *Connective Tissue.*

Exp.	Method of ash	Ash % of wet	SO_4 %	PO_4 %
29	NH_4NO_3	·516	—	—
30	,,	·503	—	—
31	,,	·628	—	—
31 B	,,	—	·0867	·0809
32	Heat	·328	·0814	·0657
33	Heat (ether)	·318	—	·0916
34	,, ,,	·382	·0757	·0796
Mean	NH_4NO_3 =	·549	·0813	·0797
	Heat =	·343		

The combined result of the three series of experiments is therefore

TABLE V.

Tissue	Ash, % of wet	SO_4 %	PO_4 %	H_2O %
Median Nerve	·985	·0110	·768	69·81
Splenic Nerve	·914	·0644	·349	81·22
Connective Tissue	·549	·0813	·0797	81

The question now to be considered is to what extent these figures represent the electrolytes in the tissues. The answer is most easily supplied with regard to the SO_4 and PO_4 determinations. These figures could conceivably be erroneous in two directions. It is possible that sulphur and phosphorus organically combined might be oxidised and appear as sulphate and phosphate, so rendering the figures too high, and it is possible (and the possibility cannot be dismissed off hand) that preformed sulphate might be decomposed and volatilise as SO_3, rendering the amount in the ash less than that in the tissue. It would be of considerable assistance in the solution of these questions to know the total S and P present in the tissues. This would give at least the maximum possible amount of SO_4 and PO_4 and as a matter of fact the determination yielded some information in addition to this. Three further series of experiments were therefore undertaken.

Method. From ·5 to 1 gram of the dry nerve or tissue were heated in a sealed Carius tube with 2·5—3 c.c. of fuming HNO_3, for about 6 hours at 250° C. The tube was then unsealed, the contents washed out, about 3 grams NaCl added and the contents taken to dryness on a water bath. The residue was then dissolved in water, HCl added, and the estimation of SO_4 and PO_4 carried out as before.

While this method is not so expeditious as that by the use of sodium peroxide, it serves very well where small quantities of tissue are to be dealt with. Some preliminary observations of sulphanilic acid gave the results in four successive experiments:

	1	2	3	4
% S	19·0	19·3	18·8	19·1

the theoretical percentage of the pure substance being 18·5.

At the conclusion of the series three successive experiments on the same sample of urine gave the results:

	1	2	3
% SO_4	1·51	1·53	1·52

showing that under the conditions and with the quantities used the figures might be relied on as correct as far as the 3rd significant figure.

The results in the tables are given as before as percentages of the original wet weight of the tissue.

SERIES IV. *Median Nerve.*

No. of exps.	S as SO_4 %	P as PO_4 %	Notes
2	—	·781	Dried over H_2SO_4.
2	·337	·911	,, ,, ,,
8	·341	·668	,, ,, ,,
4	·397	·763	Dried over $CaCl_2$.
Mean of 16 exps. on 4 animals	·358	·781	

SERIES V. *Splenic Nerve.*

No. of exps.	S as SO_4 %	P as PO_4 %	Notes
1	·305	·344	Dried over H_2SO_4.
2	·382	·402	Dried over $CaCl_2$.
2	·304	·467	,, ,, ,,
2	—	·403	,, ,, ,,
Mean of 7 exps. on 4 animals	·331	·404	

SERIES VI. *Connective Tissue.*

No. of exps.	S as SO_4 %	P as PO_4 %	Notes
1	—	·125	Dried over H_2SO_4.
1	·258	·134	Dried over $CaCl_2$.
1	·262	—	—
Mean of 3 exps. on 3 animals	·260	·130	

These experiments are tabulated so as to show the considerable difference that exists between different animals, which is masked in the case of the ash experiments.

Combining these results with those of the Series I, II, III, we obtain Table VI.

TABLE VI.

Tissue	A Total S as SO_4 %	B SO_4 in ash %	$\frac{B}{A}\times 100$ *i.e.* percentage of possible SO_4 in ash	C Total P as PO_4 %	D PO_4 in ash %	$\frac{D}{C}\times 100$ *i.e.* percentage of possible PO_4 in ash
Median Nerve	·358	·0110	3·1	·781	·768	98·3
Splenic Nerve	·331	·0644	19·5	·404	·349	86·4
Connective Tissue	·260	·0813	31·3	·130	·0797	61·3

We would like to direct special attention to this table. Leaving out all hypotheses it shows that in three different tissues the "total ash" represents a different thing in each case. In the median nerve only 3 % of the possible sulphate appears in the ash, in the splenic 19·5 %, in connective tissue 31 %, and the phosphate follows the inverse rule, 98 %.

appearing in the median ash, 86 %, in the splenic, 61 %, in the connective tissue. The reason for this divergence forms no part of the subject of this paper, the only point that is of interest, if it is desired to show the relation of the various electromotive phenomena to the physical and chemical states, is the question how far the amount of sulphate and phosphate in the ash gives a measure of the electrolytes in the original tissue. It seems to us that it gives no measure at all. Take for example the median nerve. As practically all the phosphorus in the tissue appears in the ash, it follows that the latter contains not only the inorganic phosphate but also the phosphorus that had previously been combined as lecithin and other bodies, which are not electrolytes and from which phosphates cannot be split off by any "ion-protein" hypothesis. The case of the sulphate is but little better, and if the demonstration is not at present so complete the combination of the results obtained above with those of Ellison shows certain curious discrepancies which can be best accounted for by supposing that the sulphate in the ash of at least the connective tissue is also of double origin. The conclusion that we have come to is, therefore, that the analysis of total ash is of very little assistance to us in the problem with which we are concerned.

Conclusions.

In the horse the ash of the median nerve is ·985 % of the original weight of the nerve, that of the splenic nerve ·914 %, that of connective tissue ·549 %.

The sulphate in the ash, reckoned as a percentage of the original weight of the tissue, is for the median nerve ·0110 %, for the splenic nerve ·0646 %, for connective tissue ·0813 %. Similarly for phosphates, the percentage in the ash of median nerve is ·768 %, of splenic nerve ·349 %, connective tissue ·0797 %.

The total sulphur in the tissue, expressed as SO_4, is for the median nerve ·358 %, for the splenic nerve ·331 %, for connective tissue ·260 %.

Total phosphorus expressed as PO_4 is for the median nerve ·781 %, splenic nerve ·404 %, connective tissue ·130 %.

The ash of the median nerve contains therefore 3 % of the possible sulphates and 98 % of the possible phosphates, the ash of the splenic nerve 19·5 % and 85 %, that of connective tissue 31·3 % and 61·3 %.

There is good reason to suppose that in no case do these percentages in the ash represent the amount of the electrolytes in the original tissue.

THE NATURE OF OXYHÆMOGLOBIN, WITH A NOTE ON ITS MOLECULAR WEIGHT.

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(*From the Physiological Laboratory, Cambridge.*)

In the present paper we propose (1) to set forth some additional evidence for supposing that the union of oxygen with hæmoglobin is a chemical one, (2) to press this supposition to its logical conclusion and on thermodynamical principles deduce the heat generated when one molecular weight of hæmoglobin unites with oxygen, (3) by actual determinations of the heat produced by the union of one gram of hæmoglobin with oxygen, to calculate the molecular weight of hæmoglobin. The paper will therefore divide itself into three portions corresponding to the subjects set forth above[1].

Part I. The relation of temperature to the rate of reduction of hæmoglobin.

Until recently it was supposed universally that hæmoglobin was chemically combined with oxygen and that the reaction involved was governed by the laws of mass action. Some doubt has been thrown upon this by Wolfgang Ostwald[2] who suggested that Bohr's[3] dissociation curve for hæmoglobin could be explained by the simple hypothesis that the oxygen was held by adsorption to the hæmoglobin.

In a recent paper by Barcroft and Roberts[3] it was shown that Bohr's curve, though true for hæmoglobin crystallised out with ether and redissolved, is modified when the solution so obtained is dialysed. The curve then approximates so closely to a rectangular hyperbola that there is no difficulty in supposing the union of oxygen and

[1] The responsibility for the mathematical portion of the work rests with Hill and for the oxygen estimations with Barcroft.

hæmoglobin in salt free solutions to be represented by the simple chemical formula

$$Hb + O_2 \rightleftarrows HbO_2.$$

But inasmuch as the adsorption formula contains two constants it is capable of great adaptation and it is not impossible to draw an adsorption curve with which the determinations of Barcroft and Roberts would not conflict. Some fresh evidence on the subject is therefore desirable.

If nitrogen be bubbled at a constant rate through hæmoglobin solution the velocity of the reduction of oxyhæmoglobin is very much greater at 38° C. than at 18° C. The experiment was carried out in the following way.

A solution of oxyhæmoglobin was made, dialysed and concentrated with the pump in the manner to be described later by Barcroft and

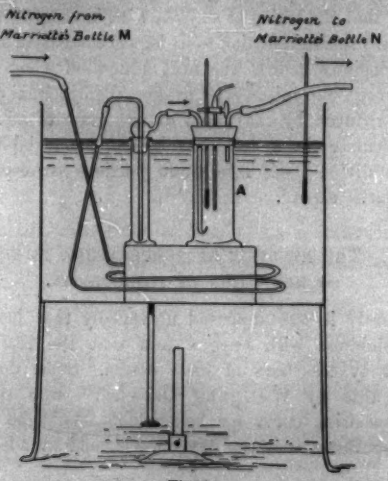

Fig. 1.

Roberts[1]. About 200 c.c. was placed in a cylindrical glass vessel, Fig. 1. This quantity rather more than half filled the vessel and was covered with a layer of oil. The vessel was fitted with a rubber cork through which three tubes led; one, through which the nitrogen entered, went to the bottom of the vessel and then turned upwards and was drawn off

[1] Cp. This *Journal*, *infra*, p. 429.

to a fine point; the second was made of capillary glass tubing and was fitted with a 3-way tap, and this served for the abstraction of samples of hæmoglobin; the third, which only just went through the cork, served as an exit tube for the nitrogen. A thermometer also went through the stopper.

The vessel was placed in a large water bath. In this bath there was also a thermometer and a wash bottle containing pyrogallic acid through which the nitrogen bubbled on its way to the vessel *A*. The nitrogen was delivered from a Marriotte's bottle (*M*) of about 15 litres capacity, and after passing first through a coil of metal tubing in the bath, then through the pyrogallic acid and the hæmoglobin, it was driven into another Marriotte's bottle (*N*) of the same capacity as the first. To set up a flow of nitrogen, water was allowed to enter *M* from a constant high level and to escape from *N* at a constant low level, thus the stream of gas went at a constant rate. The Marriotte's bottles are not shown in the figure. A constant temperature was maintained in the bath to within a fraction of a degree by a Bunsen burner.

The samples of hæmoglobin were withdrawn with a cylindrical 1 c.c. pipette graduated in $\frac{1}{100}$ of a c.c. and analysed in the differential blood gas apparatus of Barcroft and Roberts.

To compare the rates of reduction of oxyhæmoglobin at 18° C. and 38° C. the experiment runs the following course. The bath is started at 18° C.; water, equal in volume to the hæmoglobin solution which we were about to study, was placed in the glass vessel *A* and the bubbling was allowed to commence. When the pressures in various parts of the apparatus had so adjusted themselves that the nitrogen bubbled at a constant rate, the rubber portions of the tubing were clipped, the stopper was taken out of the vessel *A*, the solution of oxyhæmoglobin which had been thoroughly shaken with air was put in instead of the water, some oil was placed on the top of the hæmoglobin and the nitrogen was allowed to bubble through the hæmoglobin. The first sample of hæmoglobin was withdrawn for analysis at the end of 35 minutes. It was analysed by the differential method and found to be 94% saturated. The nitrogen was then stopped. The data furnished by this experiment are recorded in Fig. 2 in which the percentage saturation is plotted vertically and the time from the commencement of the passage of nitrogen through the hæmoglobin solution is plotted horizontally. *AD* on the curve corresponding to Exp. 1 represents the portion of the experiment which we have just described. At this point the temperature of the hæmoglobin was

raised as rapidly as possible to 38° C. The time which this process took is not included in the figure. The nitrogen was once more allowed to pass. By counting the bubbles which entered the Marriotte's bottle *N*, observations were made from time to time to satisfy ourselves that the rate of bubbling was constant. The figure from *D* to *E* represents the subsequent course of the experiment; the actual determinations are shown by encircled points. By a calculation, the basis of which is

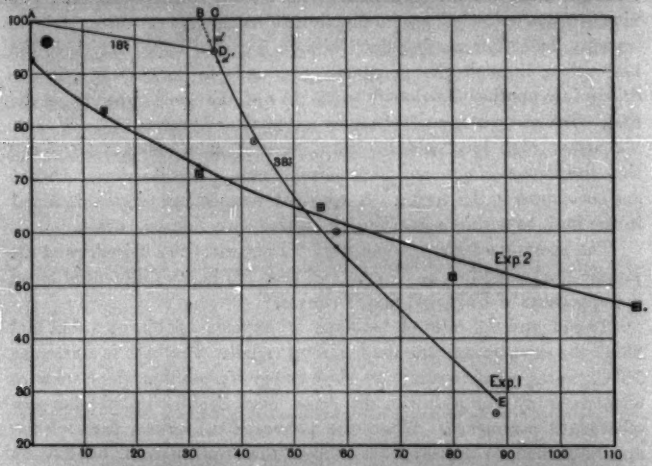

Fig. 2. Curves representing the calculated degree of dissociation at any time in Exps. 1 and 2. Percentage saturation plotted vertically, time in minutes horizontally. The points represent the actual observations.

given below, it is possible to exterpolate the curve *ED* as far as *B* and thus to discover *BC*, the length of time which would have been taken to reduce the hæmoglobin from 100 % to 94 % saturation at 38° C. This is 2·6 minutes. The velocity of the reduction is therefore 35/2·6 = 14 times as great at 38° C. as at 18° C. Over a range of 10° C. the temperature coefficient will be the square-root of what it is over 20° C. or 3·7. The limits of error in the above experiment are necessarily very considerable. The percentage saturation instead of being 94 % might be 92 % or 96 %, in which case *D* would be moved correspondingly down or up *DC* to *d'* or *d* respectively. There would be a corresponding movement of *B*. The experiment shows with

certainty however that the temperature coefficient over a range of 10° C. is between the limits 3·5 and 4·6. This high temperature coefficient is strong evidence in favour of the hypothesis of chemical combination between O_2 and hæmoglobin.

The actual rate of reduction of any hæmoglobin solution will of course depend upon the strength of the solution and the rate of the stream of nitrogen, but if the action is a chemical mass action the curve must be capable of being represented by a formula which is obtained by the following calculation.

If $[O_2]$ be the concentration of oxygen in the solution we have the equation

$$HbO_2 \rightleftarrows Hb + O_2.$$

Now at the surface of any bubbles of gas we have a diffusion of gas into the bubble from the solution and its rate of diffusion is proportional to $[O_2]$.

Hence if the rate of bubbling is always the same the rate of loss of oxygen from the solution is proportional at any moment to $[O_2]$.

Now let $100x/a$ be the percentage saturation, x the number of molecules of HbO_2 present (assuming the Hb to be present as molecules), a the total number of molecules present whether as Hb or HbO_2.

The amount of O_2 present $= x +$ the amount in simple solution. The latter term is small compared with the first and may be neglected.

$\therefore\ -\frac{dx}{dt} =$ the rate of loss of oxygen from the solution $= \lambda[O_2]$.

Now the laws of mass action give us

$$-\frac{dx}{dt} \propto k\,[HbO_2] - k'\,[Hb][O_2],$$

$$i.e.\quad -\frac{dx}{dt} = k\mu\,[HbO_2] + \frac{\mu k'}{\lambda}[Hb]\frac{dx}{dt},$$

$$i.e.\quad -\frac{dx}{dt}(\lambda + \mu k'\,[Hb]) = \mu k \lambda x,$$

$$i.e.\quad -\frac{dx}{dt} = \frac{\mu k \lambda x}{\lambda + \mu k' a - \mu k' x},$$

$$i.e.\quad +\frac{dx\,(\mu k' x - \lambda - \mu k' a)}{\mu k \lambda x} = dt,$$

$$i.e.\quad dx\left\{\frac{k'}{k\lambda} - \frac{\lambda + \mu k' a}{\mu k \lambda}\frac{1}{x}\right\} = dt,$$

and integrating,

$$\left[x\frac{k'}{k\lambda} - \frac{\lambda + \mu k' a}{\mu k \lambda}\log_e x\right] = t,$$

where the square brackets signify "between the limits of integration,"

$$i.e.\quad -(x_0 - x)\frac{k'}{k\lambda} + \frac{\lambda + \mu k' a}{\mu k \lambda}\log_e \frac{x_0}{x} = t.$$

Thus if y_0 be the initial percentage saturation and y the percentage saturation at any time, this may be written

$$\alpha \log_{10}\frac{y_0}{y} - \frac{\beta}{100}(y_0 - y) = \gamma t.$$

The constants α, β, γ involve the rate of bubbling through, the velocity constants, and the temperature.

[α is $(\lambda+\mu k'a)\log_e 10$, β is $\mu k'$, γ is $\mu k\lambda$.]

Exp. 2 and a part of Exp. 1 show the actual correspondence between the formula and the observed results. In each case the temperature was 38° C. but the bubbling was much more rapid in the case of Exp. 1 than in that of Exp. 2: hence the reduction was more active in the former case. Of the curves shown in Fig. 2 the portion *DE* of Exp. 1 and the whole of Exp. 2 are curves calculated from the above formula. The points which are actually observed lie very close to these curves. Of these two curves, much the most stress is to be laid upon that of Exp. 2 (so far as this aspect of the experiment is concerned). In drawing the curve it is necessary to assume the correctness of the first and two other points in order to decide the constants. Now it might happen that three given points could not be made to lie on any curve which was drawn from the above equation; this however is practically not very likely to be the case, so that the real point at issue is whether the remaining determinations do or do not agree with the curve which at once satisfies the formula and passes through three points. Clearly then the greater the number of determinations the more exacting is the test. In the case of Exp. 1 we only have four determinations on the curve in all. The results so far as they are depicted are reliable: the experiment was carried on longer than is shown and it then appeared that the continued incubation set up an appreciable degree of bacterial action. This was discovered from the fact that after the blood gas determination had been made the fluid in the manometer of the blood gas apparatus gradually crept upwards on the side in which the incubated solution was placed[4].

In Exp. 2 we modified the method of making our hæmoglobin so as to insure greater freedom from bacteria.

The blood was centrifugalised and the corpuscles were washed three times with Ringer's solution. The mass of moist corpuscles was treated with ether to crystallise the hæmoglobin and the thick paste of ethereal crystals was placed in a dialysing tube and dialysed for two days with frequent changing of the distilled water. On account of the ether present, the process of dialysis will have started with the whole contents of the dialyser free from bacteria, and as the ends were closed there was very little opportunity for germs to enter. At the end of the dialysis all trace of ether had gone and the solution was sufficiently

strong to serve for the experiment without concentration. It was centrifugalised to rid it of any solid particles present, either undissolved crystals or traces of precipitated protein from the stroma. At the end of the experiment the samples of solution used in the apparatus were allowed to remain for four hours in the bottles in which they had been analysed: none of them showed any sign of absorbing the oxygen in the bottles.

The results of Exps. 1 and 2 are as follows:

Exp. 1.	Time (minutes) measured from *D*	0		7·5	23		53
	Percentage saturation observed	94		77	60		26
	Percentage saturation calculated	94		79	58		31
Exp. 2.	Time (minutes)	0	14·5	32	55	80	114
	Percentage saturation observed	92·5	83	71	64	55	46
	Percentage saturation calculated	92·5	82	72·5	63	51·5	46

It is clear then that in the case of two solutions, prepared somewhat differently, of different strengths and through which the nitrogen ran at very different velocities the course of the reduction followed a complicated formula based upon a conception of oxyhæmoglobin as a chemical compound.

We feel justified then in regarding it as such for the purposes of the succeeding experiments

Part II. Determination of the heat of combination of the gram-molecule of hæmoglobin with oxygen.

On the assumption that the action is of a chemical nature we have used the laws of thermodynamics to give us a value for the heat of combination of the gram-molecule of hæmoglobin with O_2.

Let us assume that the molecule of hæmoglobin contains n atoms of iron, and therefore combines with n molecules of oxygen. The reaction would be:

$$(Hb)_n + nO_2 \rightleftarrows (HbO_2)_n.$$

So that for the equilibrium

$$\frac{[(HbO_2)_n]}{[(Hb)_n][O_2]^n} = K.$$

If $n = 1$ this gives a rectangular hyperbola for the dissociation curve. Barcroft and Roberts[8] obtained six points which within the limits of error lay on such a curve: but this is not conclusive proof of the simple value of n, for it would be possible on the assumption that $n = 2$ to draw

a curve with a different value of K which will satisfy the observations almost as well.

From the laws of thermodynamics we know that K varies with the temperature according to the equation:

$$\frac{1}{K}\cdot\frac{dK}{dT}=\frac{-q}{RT^2} \quad \text{.....(A),}$$

where T is the absolute temperature and q the heat given out by the union of one gram-molecule of hæmoglobin with oxygen[8]. Let us now assume that q remains constant over the range of temperature under consideration, an assumption which is commonly made.

Then integrating the above equation between the limits T and T_0:

$$K=K_0 e^{\frac{-q}{R}\cdot\frac{T-T_0}{TT_0}} \quad \text{.....(B),}$$

where K and K_0 are the equilibrium constants at the absolute temperatures T and T_0. Two points arise out of the equation.

(1) If any two values be taken for T and T_0, are the corresponding values for K always such as to give the same value for q? If so the integration of equation (A) is justifiable.

(2) What is the actual value of q?

We determined to compare the values of K at different temperatures. *If the oxygen tension be kept constant* during the whole experiment K may be taken as proportional to $\frac{\text{Percentage saturation}}{100-\text{percentage saturation}}$.

The problem therefore resolves itself into a determination of the percentage saturation of a given solution of hæmoglobin at different temperatures over as large a range as possible.

Our method of doing this was as follows. A litre flask was used for exposing the hæmoglobin solution to the gas. The strength of the hæmoglobin solution was about 10%: the 10 c.c. used were therefore capable of liberating about 1·3 of O_2 and thus producing a maximum difference of 1 mm. of mercury in the oxygen pressure of the gas in the flask. In reality the difference of oxygen pressure was never so great because (1) the hæmoglobin was never completely reduced and (2) at each increase of temperature a cubic centimetre or more of solution was taken out so that at the end of the experiment there was virtually no hæmoglobin solution left in the apparatus.

The apparatus is shown in Fig. 3. The flask is immersed in a water bath which consists of a saucepan into the lid of which a short tube has been soldered to accommodate the neck of the flask. The bath is

brought to the required temperature and the apparatus vigorously shaken, care being taken to maintain the thermometer reading steady.

In order to take a sample of hæmoglobin for analysis the lid is taken off the bath and rapidly inverted so that the mouth of the flask points downwards and the base upwards. The hæmoglobin solution then falls

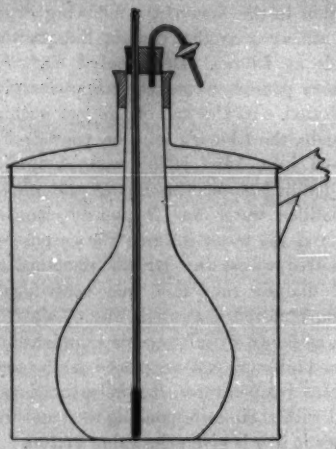

Fig. 3.

into the neck of the flask where only a small surface is exposed to the atmosphere of the flask; this precaution is necessary in order to prevent the change in temperature caused by taking the flask out of the bath from affecting the saturation of the oxyhæmoglobin. A small piece of rubber tubing of fine bore is attached to the capillary glass tubing which passes through the cork of the flask. As we have worked from lower temperatures to higher ones, mere opening of the tap on this tube will allow a drop of hæmoglobin solution to be driven out, thus clearing the dead space of the tubing. The end of a 1 c.c. pipette is put into the tube and a sample taken out for analysis.

The hæmoglobin was exposed to an atmosphere consisting of oxygen and nitrogen, the former exerting a pressure of 10 mm. of mercury or less. The advantage of a very low oxygen pressure is that it allows of a large range in the percentage saturation at different temperatures.

The flask was almost filled with distilled water and inverted over water. It contained therefore a little air. The water was then displaced by nitrogen from a cylinder. The cork was put into the neck of the bottle under water. About 10 c.c. of hæmoglobin solution were then forced in through the capillary tube, after which the tap was opened momentarily to equalise the pressure within and without the flask.

The hæmoglobin in the first of the following experiments (Exp. 3) was made in the same way as that used in Exp. 2, whilst that used for Exp. 4 was made as follows. Defibrinated dog's blood was centrifugalised, the serum poured off and the corpuscles given two washings with Ringer's solution. At the end of the last washing the blood was centrifugalised again, the Ringer's solution poured off and the mass of corpuscles dialysed in a tube which was rhythmically raised and lowered in the distilled water throughout the whole period of the dialysis. The distilled water was frequently changed. As the salts passed outwards and the water inwards the corpuscles laked and the hæmoglobin dissolved whilst the stroma remained undissolved. At the end of the dialysis the fluid was centrifugalised: after the sediment had been removed a solution was obtained which contained no solid particles, as shown by microscopic examination.

In the subjoined table (Exp. 3) are placed (*a*) the actual % saturations observed at different temperatures, (*b*) the saturations giving values of K, which inserted with their corresponding temperatures into the above formula (B), satisfy it if q is given the value 27,700.

Exp. 3. Temperature	16°	25°	32°	38° C.
a, % saturation (observed) ...	96	89	77	52
b, % saturation (calculated) ...	97	89	74	54
K, corresponding to *b*, ∞ ...	33	8	2·8	1·17

A similar table is given for Exp. 4. Here the K's are calculated with the value 28,700 for q.

Exp. 4. Temperature	16°	24°	32°	38°	49° C.
a, % saturation (observed) ...	92	71	37	18	6
b, % saturation (calculated) ...	90	71	41	22	6
K, corresponding to *b*, ∞ ...	9	2·4	·69	·28	·06

From the above figures we conclude that:

1. Since the observed saturations are all within the maximum error (5 %) we are justified in regarding q as a constant quantity for any given solution of hæmoglobin at whatever temperature it is observed, and that therefore the equation (A) may be integrated.

2. Since two solutions prepared (*a*) by crystallisation with ether and (*b*) without the use of ether or alcohol, give values for q (27,700 and 28,700) which are so close as to be within the probable limits of error, the value of q may be regarded uniformly as 28,000 calories.

3. It is now possible, with the assumption that $n=1$, when the hæmoglobin molecule is represented by [Hb]n, to draw the dissociation curve for hæmoglobin at any temperature. In making this assumption we are merely anticipating the proof given in the last part of the present paper.

The following are the principles on which such a curve is drawn:

The formula for the dissociation when $n=1$ is:

$$Hb + O_2 \rightleftarrows HbO_2.$$

If the total concentration of hæmoglobin be 100 and the percentage present as reduced hæmoglobin be Cr and the oxygen pressure p, the laws of mass action give

$$(Cr)\left(\frac{1}{K}+p\right)=\frac{100}{K}.$$

If then we call (Cr), y; and $\left(\frac{1}{K}+p\right)$, x we arrive at the fact that $xy=$a constant (at any particular temperature) which involves K. If values of y are plotted vertically downwards and of x horizontally to the right, the curve is a hyperbola in which the distance of any point from the horizontal axis represents the percentage of reduced hæmoglobin present (or $100-$% HbO_2) and any distance measured horizontally from the vertical axis represents, not the pressure, but $\left(\frac{1}{K}+p\right)$.

For 38° C. or 311° absolute:

$$\therefore\ xy=800=\frac{100}{K},\qquad \therefore\ \frac{1}{K}=8.$$

When the hæmoglobin is entirely reduced $Cr=100$ and p will therefore $=0$ while $x=8$.

If therefore the numerical quantities be represented on the drawing in millimetres, and a vertical line be drawn 8 mm. to the right of the vertical axis, then the pressures are measured from this line, and the hyperbola will pass through it at a point 100 mm. from the top; this point is the origin of the dissociation curve.

For any other temperature T, it is necessary to find a fresh value for K; having found it we get a fresh value for $xy\left(=\frac{100}{K}\right)$ and as before the line representing zero pressure is $\frac{1}{K}$ mm. to the right of the vertical axis.

The fresh value for K comes directly from the formula

$$K=K_{38}\,e^{\frac{-q}{2}\cdot\frac{T-T_{38}}{T.T_{38}}},$$

where $K_{38}=\frac{1}{8}$, $\log e=\cdot434$, $q=28{,}000$ and $T_{38}=311$.

In the present research the value $xy=800$ for 38° has received some experimental verification. In the table corresponding to Exp. 3, the calculated saturation of oxyhæmoglobin is 54 %, i.e. there is 46 % of

reduced hæmoglobin. The tension of the oxygen in the flask should therefore have been $\frac{800}{46} - 8$ mm. $= 9{\cdot}4$ mm. Direct analysis showed it to be $9{\cdot}7 \pm 1{\cdot}0$ mm.

In Fig. 4 we show dissociation curves of hæmoglobin drawn on the basis of the values of K in the above tables.

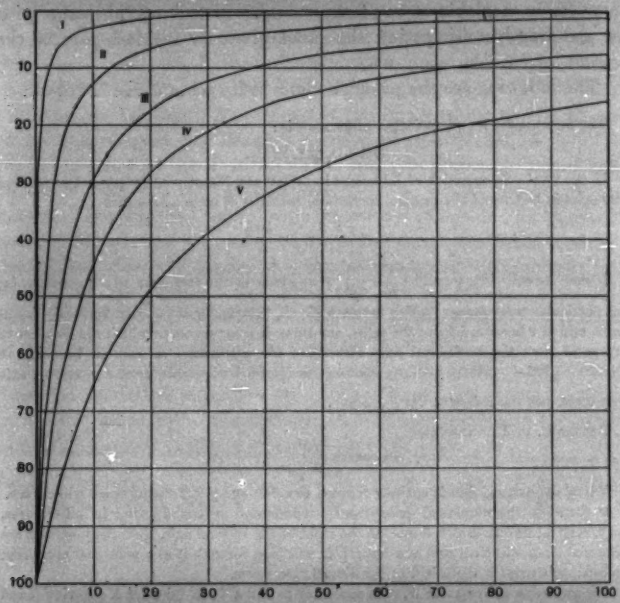

Fig. 4. Dissociation curves I, II, III, IV and V correspond to 16°, 25°, 32°, 38° and 49° C. respectively. Oxygen tension plotted horizontally, percentage saturation of hæmoglobin vertically.

Part III. Determination of molecular weight.

If H be the amount of heat given out when one gram of hæmoglobin unites with oxygen and q the amount when one gram-molecule unites with oxygen, the molecular weight is q/H. A value for q has been obtained above: we propose to describe the method by which we have determined H.

The hæmoglobin solution was made as in Exp. 4. The oxygen was pumped off in the blood gas pump. The receiver of the pump was then filled with nitrogen from a cylinder; the pressure of the gas in the receiver was somewhat in excess of the atmosphere pressure. A 100 c.c. pipette containing a little oil was placed in the position of the burette (see Fig. 2. Barcroft and Roberts. This *Journal*, infra p. 429): on opening the tap the pressure drove the hæmoglobin solution into the pipette, the surface in the pipette being covered with a film of oil. By closing the ends of the pipette the solution of reduced hæmoglobin could be kept unchanged for a short time.

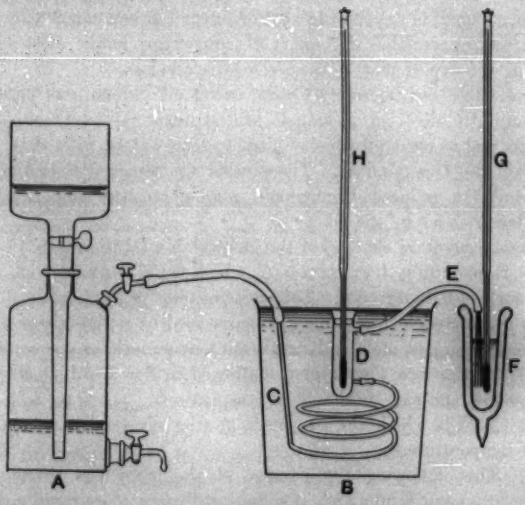

Fig. 5.

The oxygen for the experiment was taken from a gas holder, Fig. 5 *A*, which had previously been filled from a cylinder over distilled water. The oxygen was therefore saturated with aqueous vapour and changes of temperature in the hæmoglobin solution did not arise from evaporation when the oxygen was bubbled through it, nor was the gas cooled by violent decompression. The gas passed through a long coil of flexible metal tubing immersed in a large water bath. The tube

communicates with a short glass tube, also in the water bath, which contains the bulb of a Beckmann's thermometer. The tube is closed but for the apertures for the entrance and exit of the oxygen. The gas leaves *D* by a piece of rubber pressure tubing; into the end of this are fixed, by means of plasticine, four glass tubular filaments sufficiently long to reach to the bottom of the calorimeter which is a Dewar's flask *F*.

For an hour or so before the commencement of the experiment, the pipette containing the reduced hæmoglobin was immersed in the bath *B*, so that there might be as little difference as possible between the temperature of the hæmoglobin and that of the oxygen entering it, though, so small is the specific heat of oxygen as compared with water, that a very appreciable difference of temperature might exist without vitiating the experiment. (See also controls below.)

Five cubic centimetres of cedar wood oil (which was chosen in preference to olive oil inasmuch as the latter is apt to be acid in reaction, and to clove oil which unites to some extent with water) were placed in the Dewar's flask. The pipette was removed from the water bath and the reduced hæmoglobin allowed to run into the flask, protected from air by the oil.

The temperature changes in the hæmoglobin solution were observed with a Beckmann's thermometer *G*, which could be read with a lens from some little distance. The solution was stirred with a small hollow globe made of very fine glass suspended by a glass filament.

The experiment may be divided into four periods.

(1) That before the oxygen is allowed to flow in which there will be a gradual and regular change of temperature.

(2) That in which the oxygen is flowing: in this there is a rapid rise of temperature.

(3) That following the passage of the gas. The thermometer, getting into equilibrium with the fluid, still rises more rapidly than in period (1),—also possibly the hæmoglobin itself requires an appreciable time to get into equilibrium with the oxygen dissolved in the fluid.

(4) The period subsequent to the chemical action in which the change of temperature should be the same as in period (1).

To calculate the rise of temperature due to the chemical action we subtract from *T*, the rise during periods (1) and (2), *t'*, the change which would have taken place during the same period of time had no oxidation taken place. This is calculated from periods (1) and (4).

The temperature was noted every minute. The results of Exp. 5, which is typical, may be abridged as follows:

Period	Time	Rise of Temperature	Rise of Temp. per min.
I	4 min.	·013° C.	·00325° C.
II	9 min.	·158° C.	·0262° C.
III	1 min.	·012° C.	
IV	4 min.	·013° C.	·00325° C.

$T = ·162°$ C. $t = 10 \times ·00325 = ·032°$ C. $T - t = ·130°$ C.

That bubbling oxygen through distilled water under similar circumstances does not cause a rise of temperature which can be read by the thermometer is shown by the following Exp., in which water was substituted for hæmoglobin solution.

Period	Time	Rise of Temperature	Rise per min.
I	3 min.	− ·001° C.	− ·0003° C.
II	4 min.	− ·001° C.	− ·0002° C.
III	non-existent		
IV	4 min.	− ·004° C.	− ·001° C.

Taking the nett rise of temperature in Exp. 5 as 0·13° C. it is necessary to calculate the actual heat production; several circumstances have here to be considered. (1) The specific heat of the hæmoglobin solution differs from that of water. (2) The oil, the flask and the thermometer are heated as well as the solution. The best way of treating these factors is to find the water equivalent of the flask and its contents. For this purpose a known quantity of water at a known temperature was added and its heating effect observed.

Thus the addition of 5·66 c.c. of distilled water at 33° to the flask which contained 66 c.c. of solution, five of oil, the thermometer and the stirrer raised the temperature 1·110° C. The final temperature was 17°; from which it follows that 90·6 calories raised the equivalent of x grams of water 1·110° C.; therefore $x = 81·6$ c.c. The addition of a second 5·66 c.c. of water (after making the necessary deduction for the increased volume of fluid) gave a value of 84·7: mean value 83·1.

This was slightly too large as the added fluid heated more glass than was occupied by the original 66 c.c.; to correct for this we have assumed that the flask and the thermometer were cylindrical, neglected the base of the flask and the oil and supposed that the amount of glass heated by the 5·66 c.c. of water bore the ratio of $\frac{66 + 5·66}{66}$ to that of the glass heated by the oxygen. This corresponds to a deduction of approximately 1 calorie from the figure given above, leaving 82 c.c. as the water equivalent of the calorimeter and its contents.

The heat given out by the oxidation is therefore $82 \times 0{\cdot}13 = 10{\cdot}66$ calories, i.e. $\frac{10{\cdot}66}{66} = {\cdot}162$ calorie per c.c. of the hæmoglobin solution.

The other measurement required was the mass of hæmoglobin oxidised in 1 c.c. of the solution.

At the commencement of the experiment the hæmoglobin may not be entirely reduced, at the end it is not entirely oxidised, so that the total amount of hæmoglobin in the solution bears no relation to the amount which has been oxidised.

Under these circumstances we adopted the method of measuring, with the differential blood gas apparatus, the difference between the amount of oxygen in equal volumes of the hæmoglobin solution as it was before and after the oxidation and deduced the amount of hæmoglobin oxidised. This method has one disadvantage, namely that it assumes a knowledge of the precise ratio between the oxygen and the hæmoglobin —a point about which there is much difference of opinion. For the purposes of the following calculations we have taken Hüfner's figure as being correct—1·34 c.c. of oxygen = 1 gram of hæmoglobin. We put forward no brief for this figure and will not discuss its merit since the use of a slightly different one will not alter the essential argument of our research.

The method which we have adopted has advantages over the alternative one (that of completely oxidising the hæmoglobin and subsequently drying and weighing it) which far outweighed the limited uncertainty respecting the relation of the oxygen to the hæmoglobin.

1. It would be a very difficult and laborious matter to reduce completely—as opposed to approximately—the large mass of hæmoglobin solution necessary for the experiment. Moreover the solution so reduced would be spoiled if it came in contact with even the smallest bubble of air after the reduction.

2. The process of oxidising it completely would be a lengthy one, whilst that of oxidising say two-thirds of the hæmoglobin is a rapid one. The advantage of cutting periods II and III of the experiment as short as possible is very great, since so doing diminishes t in relation to T.

3. If the hæmoglobin is only 60 or 70 % saturated at the end of the experiment the final tension of oxygen in the fluid is but a few mm. and therefore the quantity in solution is practically *nil*. This fact avoids any correction for the heat of solution of the oxygen.

4. If the hæmoglobin were weighed, or even the iron estimated, it

would be necessary to make the very dangerous assumption that there was no methhæmoglobin present.

In the case of Exp. 5, 2·3 c.c. of solution were taken before the commencement of period (1) and the same quantity after the oxidation. The analysis showed that 0·089 gram of hæmoglobin per c.c. of solution had been oxidised. H therefore $= 0{\cdot}162/{\cdot}089 = 1{\cdot}82$ calories. Two similar experiments gave 1·98 and 1·75 calories. The mean value for the heat of combination of one gram of hæmoglobin is therefore 1·85 calories. This value is greater than that given by either Torup[6] or Camis[7].

So far we have assumed in our argument that the molecule of hæmoglobin is $(Hb)_n$, where (Hb) is the least possible molecular weight. This contains one atom of iron, and n may be 1 or any higher whole number. $(Hb)_n$ then has the molecular weight

$$\frac{q}{H} = \frac{28{,}000}{1{\cdot}85} = 15{,}200 : \text{ hence } Hb = \frac{15{,}200}{n}.$$

But chemical analysis shows the least possible value for the molecular weight of hæmoglobin to be 16,669[8]: to this $\frac{15{,}200}{n}$ must be approximately equal, and n must therefore be 1. Hence the molecular weight of hæmoglobin is the least possible value 16,669. This result agrees with the determinations of Hüfner and Gausser[9], who obtained a similar molecular weight from measurements of osmotic pressure. Waymouth Reid[10] on the other hand found a value 48,000, while Roaf[11] found a value of about 32,000 for hæmoglobin in distilled water, and of 16,000 for hæmoglobin in sodium carbonate solutions. Both used the osmometer method. Roaf has as yet published only a preliminary communication.

It is not at all unlikely that a solution of hæmoglobin prepared by Bohr's method might contain aggregates of molecules, and its dissociation curve can be explained on this theory; possibly all undialysed solutions contain aggregates to a greater or less extent; this however must form the subject of a future paper.

Conclusions.

1. The velocity of dissociation of oxyhæmoglobin obeys an equation derived from the laws of mass action, and has a high temperature coefficient, increasing about 4 times for a rise of 10° C.

2. The variations of the equilibrium constant K, with change of absolute temperature T, follow the equation

$$\frac{1}{K}\cdot\frac{dK}{dT}=\frac{-q}{2T^2},$$

where q is constant and equal to 28,000 calories. From the second law of thermodynamics q is the heat of combination of one gram-molecule of hæmoglobin with oxygen.

3. The amount of heat (H) given out when one gram of hæmoglobin unites with oxygen is 1·85 calories.

4. The molecular weight of hæmoglobin in dialysed solution is the least possible value 16,669.

5. From the high temperature coefficient and from the fact that the reaction obeys several equations based upon a chemical conception of its nature, we conclude that oxygen unites chemically with hæmoglobin according to the formula $Hb + O_2 \rightleftharpoons HbO_2$.

The expense of this research has been in part defrayed by a grant from the Royal Society.

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IMPROVEMENTS IN THE TECHNIQUE OF BLOOD-GAS ANALYSIS.

By JOSEPH BARCROFT, M.A., B.Sc., *Fellow of King's College*, AND Ff. ROBERTS, *Scholar of Clare College, Cambridge.*

SINCE the publication of the differential method of blood-gas analysis the apparatus has been made more convenient in several respects.

1. The manometer is all made in one piece.
2. The bottles are egg-shaped.
3. A different method is used for raising and lowering the fluid.
4. The fluid used is oil of cloves or cedar-wood oil. The apparatus in the form now used is shown in Fig. 1.

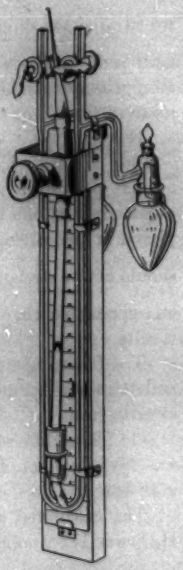

Fig. 1.

Since the manometer is in one piece of glass the method of calibration by the process previously described is cumbersome, and we have devised a new method, namely, by a direct observation of the change in the pressure produced in the manometer by the liberation of a known quantity of gas within the instrument.

Several chemical reactions may be employed for this purpose. Mention may first be made of one which we have tested and discarded, namely the liberation of CO_2 from a standard solution of sodium carbonate. This method, though theoretically good inasmuch as the reaction is complete, has two practical disadvantages, the gas comes off too rapidly which makes it very difficult to be certain of the zero reading, and it is soluble in the fluids with which it comes in contact.

Four chemical methods have been used successfully:

(1) To calibrate the instrument with blood or hæmoglobin solution against a standard pump, or

(2) Standard Haldane's hæmoglobinometer.

Little need be said concerning these methods which give very good results in the hands of experts. They have the disadvantage however that in the last resort they entail a knowledge of the accurate quantitative extraction and analysis of blood-gases with the pump.

(3) The reaction which we have found most satisfactory has been that of potassium permanganate on hydrogen peroxide in acid solution.

(4) With equal accuracy we have used barium peroxide but inasmuch as it is more difficult to prepare we regard hydrogen peroxide as the more expedient reagent. We used as a stock solution the so-called "20 volume" hydrogen peroxide; 1 c.c. of this was diluted 100 times. As hydrogen peroxide solution gradually deteriorates in strength, we titrated the dilute solution with $\frac{N}{10}$ $K_2Mn_2O_8$ immediately before we used it for analysis and again some hours afterwards. It did not lose strength appreciably in this time. For gas analysis 1·5 c.c. of the solution (sufficient to liberate about ·2 c.c. of O_2) and the same quantity of $\frac{N}{100}$ H_2SO_4 are put into each bottle, whilst approximately 0·2 c.c. of normal potassium permanganate was put into the tube contained in the stopper of one bottle and the same quantity of distilled water was placed in the corresponding tube on the other side. The sulphuric acid should not be stronger than $\frac{N}{100}$, otherwise when permanganate is added an appreciable change of pressure of the aqueous vapour in the bottle results.

The following example will show how the constant of the apparatus is determined by means of peroxide and permanganate and by control experiments.

(1) The difference of pressure produced by liberating the oxygen was 50·5 mm. of fluid × ·92 the correction T.P. and aqueous vapour = 46·4 mm.

The calculated oxygen which should be given off from 1·5 c.c. of H_2O_2 was 175 c.mm.

The constant of the apparatus is $\frac{175}{46·4} = 3·75$.

Two similar determinations gave 3·77 and 3·81; mean value of K = 3·78.

A second set of observations with somewhat weaker H_2O_2 gave 3·80 as the mean value of K.

(2) The apparatus used was one of the old pattern since, unlike the new, it could be calibrated by weighing the parts with and without mercury and calculating according to the formula:

$$V(x-y) = 2\rho\left(\frac{V}{p} + 0{\cdot}785d^2\right),$$

where V is the volume of the bottle, $V(x-y)$ that of the gas given off, 2ρ the difference in level observed, d the diameter of the tubing.

The term $\left(\frac{V}{p} + 0{\cdot}785\,d^2\right)$ being the constant of the apparatus = K.

In the present case $V = 30{,}000$ c.mm. (correct to the third figure), $p = 10{,}000$ mm. of fluid in manometer and $785d^2 = 1{\cdot}10$;

$$\therefore\ K = 3{\cdot}00 + 1{\cdot}10 = 4{\cdot}10.$$

The two values of K so obtained are not comparable since that obtained by weighing with mercury (4·10) contemplates the bottles as being empty, whilst in the other case the effective volume of the bottles was smaller by that of the amount of fluid which they contained. This in each case was 3,200 cubic millimetres. To make the constants comparable therefore $\frac{3{,}200}{10{,}000}$ should be deducted from the constant as obtained by weighing which would make it 3·78. Since varying quantities of fluid may be used in the apparatus at different times it is better to state the constant of the apparatus as if the bottles were empty and to add the correction (v/p, where v is the volume of H_2SO_4, $K_2Mn_2O_8$ and H_2O in cubic millimetres and p is the atmospheric pressure in mm. of the fluid in the manometer). In the present case then the peroxide method would give $3{\cdot}78 + {\cdot}32 = 4{\cdot}10 = K$ as the result of the first series of observations and 4·12 as the result of the second.

(3) The same apparatus was calibrated by a third method, namely a direct comparison with the blood-gas pump. Two analyses of saturated blood were made in the apparatus and two of the same blood in the pump. The pump gave 12·9 and 13·1 % of O_2, mean 13·0; deduct 0·7 % for the oxygen in physical solution and 12·3 % remains, or 1 c.c. of blood contains 123 c.mm. of oxygen. The difference of pressure in the apparatus was 30·5 and 30·0 mm., mean 30·25. The amount of

blood delivered by the 1 c.c. pipette was 0·96 c.c.; therefore 1 c.c. would correspond to 31·5 mm. The constant of the apparatus is therefore $\frac{123}{31·5} = 3·90 + ·27$ (1·5 c.c. ammonia, 1 c.c. blood, ·2 c.c. ferricyanide) = 4·17 = K.

(4) Again the apparatus was calibrated by comparison with a hæmoglobinometer. This instrument was standardised by the blood-gas pump.

Oxygen capacity of hæmoglobin in dog's blood 16·3 by pump and 16·1 as the mean of three determinations by the hæmoglobinometer.

The blood used for standardising the differential apparatus gave the following readings with the hæmoglobinometer 98·5, 99, 99, mean 99, corresponding to $185 \times 99 \times \frac{163}{161} = 18·5$ % of oxygen, 1 c.c. of blood = 185 c.mm. of oxygen. In the apparatus 0·96 c.c. of the same blood gave a reading of 50·5 mm. (× ·93 to reduce to N.T.P.). This corresponds to 48·9 mm. for 1 c.c. of blood at 0° C. and 760 mm. The value of K for bottles containing fluid was therefore $\frac{185}{48·9} = 3·78 +$ correction for fluids (2 c.c. NH_3, 1 c.c. blood, ·2 c.c. ferricyanide) ·32 = 4·10.

The values of K then are:

By peroxide method series 1, K = 4·10
series 2, K = 4·12
} mean 4·11.

By other methods

1. Direct weighing with mercury K = 4·10
2. Direct comparison with blood-gas pump K = 4·17
3. Comparison with standard hæmoglobinometer K = 4·10

} mean 4·12.

There should be no difficulty then in standardising the differential apparatus by hydrogen peroxide with sufficient accuracy to obtain a value for K which is correct to the second significant figure.

As the pump used for some of the observations mentioned above has not been described, the present opportunity may be taken of giving some account of it.

The actual pump is not original, it differs from the ordinary Tœpler in having no side tube: the tube from the valve goes direct into the chamber. It withstands the agitation of the mercury which takes place when air enters and no solicitude need arise on this account. In practice, however, the pump is never put to this strain since a preliminary exhaustion should be made with a water pump or a Geryk pump. This may be attached by flexible tubing either where the

burette is shown in Fig. 2 or to the delivery tube of the pump. The mercury is raised and lowered by the simple modification of Bohr's method described by one of us.

The drying tube is new. The sulphuric acid is placed in a test tube inclined at such an angle as to give the maximum surface without wetting either the tube by which the gas leaves or the ground stopper. The object in this particular sort of drying tube is as follows. Frequently the efficiency of the drying tube suffers by aqueous vapour forming a layer of dilute acid on the surface, this being lighter than the

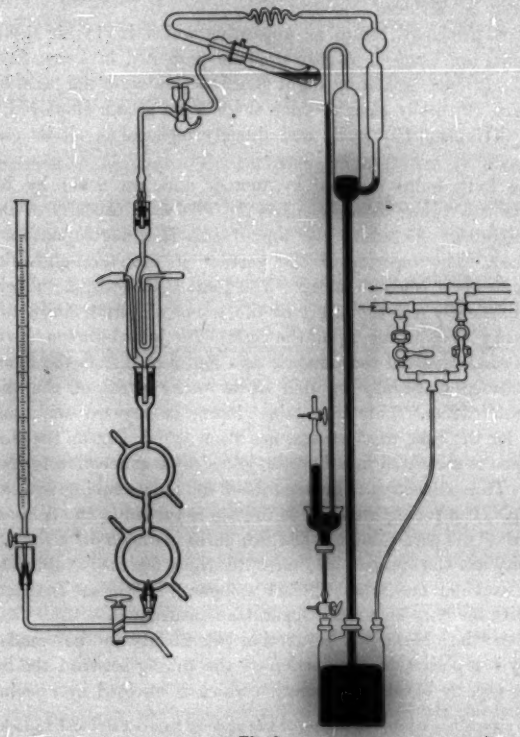

Fig. 2.

stronger acid does not mix with it. In the present form of tube, should this happen it is only necessary to rotate the test tube in order to mix the spent layer with the good acid and form a surface of strong acid both over the tube and on the fluid.

It is the receiver of the apparatus which demands rather special notice. In its construction the following principles have been observed: (1) that there shall be no ground glass joints, (2) that there shall be no unprotected rubber tubing, (3) that all the parts of the receiver shall be made of standard glass apparatus such as is illustrated in the price list of manufacturers, (4) that in no case shall the efficiency of the apparatus be sacrificed to convenience.

These principles insure an apparatus which is (1) air tight since the joints are made of rubber stoppers protected in every case with mercury, (2) not broken by slight strain or jars since the junctions are not rigid, (3) easily cleaned since there is no grease employed in the joints, (4) cheap, (5) easily and quickly replaced in all its essential parts.

The froth bulbs are two in number and are made by blowing together two "glass-ball condensers[1]." The total capacity of these is about 650 c.c. Fitted to the top of this is a double surface water condenser. The capacity of the portion of this through which the blood-gases pass is about 50 c.c. The joint is made by a rubber cork, sunk sufficiently into the orifice at the top of the froth bulbs to allow of mercury lying on the top of the cork. The joint between the double surface condenser and the pump is best understood from the figure, the parts shaded diagonally in this as in other parts of the diagram represent rubber. This like other joints is covered with mercury which, for the sake of clearness, has been omitted from the drawing. The blood is measured in a burette, joined by a connection to the froth bulbs. This connection is made of 1—2 mm. glass tubing with a three way tap. The tubing attached to the tap is turned in the flame and in the case of the part going to the froth bulbs is drawn off a little. The extremity can thus project into the bulb whilst a piece of rubber tubing on the shoulder makes an efficient stopper. This glass connection is filled with mercury before the apparatus is evacuated.

When the blood-gas estimation is about to be made this mercury is replaced, in the portion of the tubing between the burette and the tap, by blood—an operation which is effected by opening the

[1] The apparatus described in this paper was made by Messrs Baird and Tatlock, Cross Street, Hatton Garden, London, E.C.

tap of the burette and turning the three way tap in precisely the opposite direction to that shown in the figure.

The only point which remains to be described is the course taken by the water in the jackets. Cold water from the tap enters the double surface condenser at the orifice on which a piece of tubing is depicted in the drawing. Its other orifice is connected by rubber tubing to the lower orifice of the jacket of the upper froth bulbs. The water therefore circulates from the condenser to the upper bulb and goes back to the sink from the upper orifice of the same.

Hot water circulates in the jacket of the lower froth bulb, best from a tap, but if necessary it may be passed backwards and forwards from one large glass vessel to another; these reservoirs are connected to the orifices of the lower jacket by a sufficient length of rubber tubing to allow of their being raised or lowered at will. By this arrangement the blood is kept boiling continuously.

Differential Apparatus for the Analysis of 0·1 c.c. *of Blood.*

For the systematic direct determination of the oxygen capacity of the blood of human beings it is necessary to have an apparatus which will analyse blood in such quantities as may be obtained readily from a prick. This presents no difficulty since it is only necessary to reduce the size of the bottles and the bore of the tubing in the differential apparatus in order to get readings of 50 mms. for 0·1 c.c. of blood. The apparatus which we have used is represented in Fig. 3. The bottles which are of the same size are about 2 c.c. in capacity, the glass tubing is 2 mm. bore with the exception of the manometer which is about ·3 mm. bore. Such tubing is too fine for a water manometer, but is admirable when filled with cedar-wood oil. The oil finds its level, though slowly, with great certainty.

The oil is introduced into the manometer with a fine glass pipette. This is passed along the 2 mm. tubing till it reaches the manometer. The amount of oil which is judged necessary is then allowed to flow out of the pipette. Should it be found, when the oil sinks to its proper level, that too little has been taken the error may be remedied by putting a little more at the top of the manometer on one side, say the right; then with the bottle on the left side and the left tap shut place the finger on the left bottle. The rise of temperature so produced will drive the oil up on the opposite side till it joins with the fresh drop that has been put in. Should too much have been introduced at first,

the pipette is inserted till its orifice reaches the point where the tubing narrows. The oil is driven up to it in the manner described above. Some oil will run up the capillary pipette and can thus be abstracted.

In order to make an analysis about ·2 c.c. of ammonia is introduced, the quantity being exactly the same on all occasions. This solution is made by the addition of 2 c.c. of strong ammonia (s.g. ·880) to 500 c.c. of distilled water. Our custom has been to introduce 4 drops from a burette (the point of which goes almost to the bottom of the bottle, 19 such drops = 1 c.c.) into the bottle in which we are about to make the analysis and five such drops into the control bottle which will receive no ferricyanide. Blood is then put into each bottle from a carefully calibrated pipette; the one used by us delivered 0·099 c.c. The blood is then laked, care being taken not to wet the glass in the vicinity of the pouch situated about half way down the bottle. Into this pouch by means of a capillary pipette, slightly curved at the end, a drop of ferricyanide is placed on the one side. The bottles are then put in their places. The apparatus is hung on the side of a water bath into which a tap is pouring a stream of water, and the analysis proceeds as in the case of the larger apparatus. The constant of the apparatus may be determined by any of the methods which have been described.

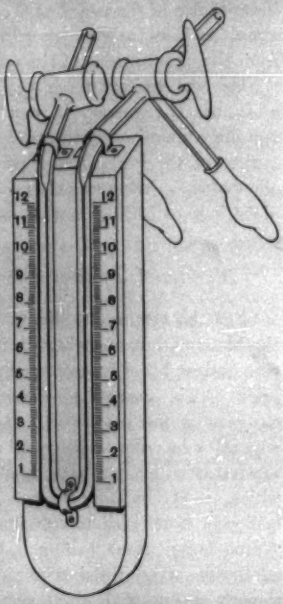

Fig. 3.

The following tests have been made with the apparatus:

(1) To determine the consistency of the apparatus three analyses of the same defibrinated blood were made; they gave the following differences of pressure 48·0, 47·3, 47·0 mm., mean 47·5, for 0·099 c.c. of blood.

(2) To determine the constant of the apparatus the same blood was analysed with the standardised hæmoglobinometer. Three hæmo-

globinometer readings gave 110, 110, 111, mean 110·3, which gave at the temperature and pressure of the experiment 1 c.c. of blood = 0·219 c.c. of oxygen. The constant of the apparatus is 0·457.

(3) To determine the constant by comparison with the larger differential apparatus, of which the calibration by a number of methods has been described, and is taken as 4·11, some defibrinated blood was analysed in each apparatus. In the large one it gave 1 c.c. of blood = ·231 c.c. of O_2. The readings in the small apparatus were ·099 c.c. of blood, which gave a difference of pressure of 49·9 and 49·1 mm., mean 49·6; ∴ 1 c.c. = 501 mm. the constant for the apparatus (without deduction for fluid) = 0·461.

(4) The following determinations of the constant were made with hydrogen peroxide and potassium permanganate, 0·458, 0·461, 0·467, 0·458, mean 0·461.

(5) In order to test the accuracy for blood containing abnormally small quantities of hæmoglobin we diluted it with known quantities of normal salt solution. The following table gives the result:

Calculated strength	100 %	66·6 %	50 %	33·3 %	20 %
Readings	49·9 49·1 } 49·6	33·0 32·5 } 32·8	24·8 25·0 } 24·9	16·4 17·1 } 16·8	9·6 9·8 } 9·7
Observed strength	—	66·3 %	50·2 %	33·9 %	19·6 %

When the apparatus is not in use the taps should be so turned as to disconnect the manometer from the rest of the apparatus.

During the measurement of the percentage saturation of blood it is desirable that the bottles should hang vertically downwards till they take the temperature of the bath, so that the blood may be the better protected by the ammonia solution. To accomplish this a hinged clip for suspending the apparatus is placed on the back of the stand.

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CONTRIBUTIONS TO THE PHYSIOLOGY OF MARINE INVERTEBRATES. I.

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(From the Port Erin Biological Station.)

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As the result of investigations by Krukenberg and others on the digestive enzymes of invertebrates it has been found that some of these appear to digest proteins in either acid or alkaline reaction. As comprehensive reviews on the enzymes [23] and digestive organs [17] of invertebrates have recently been published it is unnecessary to refer in detail to the literature.

In previous papers [13, 14] the enzymes of certain invertebrates have been investigated and it was suggested that, in accordance with the doctrine of evolution there might be amongst enzymes various stages, from those capable of acting in either acid or alkali to those which can only act in one medium. Certain experimental results support this hypothesis as the enzymes of many invertebrates have their optimum activity in acid or alkali much weaker than the optimum for pepsin and trypsin.

The results of observations on protozoa show that the food vacuole becomes first relatively acid and then goes back to a more alkaline state. Vernon [20] sums up the matter by saying that probably the acid secretion and "peptic" enzyme act at one stage of intracellular digestion and the alkaline secretion and "tryptic" enzyme at another.

If such be the case one should be able to find that during digestion the alimentary canal of those animals, which have enzymes active in both acid and alkali, would be acid at one period and alkaline at another. It is possible however that one enzyme might be purely intracellular or that there is one enzyme capable of digesting in both reactions but normally being exposed only to one. In order to investigate this point it was decided to feed animals on food dyed with various indicators. Some work on this line has already been done [5, 14], but since the method of testing the reaction by indicators was introduced by Friedenthal [9] and Salm [16] it has become possible to determine the actual hydrogen ion concentration. In carrying out such experiments it is better to mix the dye with the food and thus obtain the actual condition during digestion and not to rely upon opening an animal and then testing the contents of its alimentary canal.

A further interesting question is the reaction of the tissues of an intact animal. In reviewing the reaction of the body Henderson [4] states that the blood and all protoplasm are just on the alkaline side of the true neutral point. He seems to regard the tissues as all possessing the same concentration of hydroxyl ions but it is most likely that each tissue may have a reaction of its own. The secretion of acid by the gastric glands would seem to indicate that the cells which secrete acid may themselves be more acid than cells which do not secrete acid. The well known fact that muscle after fatigue is more acid than normal may be found to correspond to a condition of acidity during each contraction. Thus the different tissues may not only be found to possess different hydroxyl ion concentrations but physiological alterations, excitation, anæsthesia, etc., may be accompanied by an alteration of their reaction.

Methods. A selection of indicators was made from those that change colour between the degree of acidity due to decimolecular sodium dihydrogen phosphate and the degree of alkalinity due to decimolecular di-sodium hydrogen phosphate. In making this selection it was desired to use dyes which possess good "covering" power and in which the colour change was sufficiently marked to show the reaction when mixed with the food. These indicators were then tested further by placing frog tadpoles in dilute solutions of them, and if the tadpoles appeared quite active and unharmed after two days' immersion the indicator was considered non-toxic. Unfortunately the number of indicators available was not sufficient to select an ideal series for biological work.

The food was dyed in various ways. Sometimes it was sufficient to dye it in sea water to which a little of the indicator was added. Usually the food was placed in fresh water for about an hour and then transferred to fresh water containing some of the indicator in solution for eighteen hours, and finally it was placed in sea water for a short time before feeding to the animals. Occasionally the food was slit almost into two portions, the indicator placed dry between the two halves and then the two halves pressed together so that the food was swallowed with the indicator in the centre.

The following table shows the indicators used and their behaviour in phosphate solutions. The hydrogen ion concentration is taken from the paper by Salm [26].

Indicator	Acid colour	Transitional colour	Alkaline colour	Approximate normality of hydrogen ion at which colour change occurs
Di-methyl-amido-azobenzol	red	orange	yellow	1×10^{-3}
Congo red	blue	purple and brown	red	1×10^{-4}
Vesuvian brown	brown	—	yellow	1×10^{-4}
Gallein	colourless	pink	red	1×10^{-4}
Sodium alizarine sulphonate	yellow	orange	red	1×10^{-4}
Lacmoid	red	purple	blue	1×10^{-4}—1×10^{-8}
Rosolic acid	yellow	orange	red	1×10^{-6}
Litmus	red	purple	blue	1×10^{-6}—1×10^{-8}
Aniline blue	blue	—	slowly decolourised	not very definite
Neutral red	red	orange	yellow	1×10^{-7}
Alizarine	yellow	orange	red	1×10^{-8}
Phenolphthalein	colourless	pink	red	1×10^{-9}

The reaction of the sea water from several points near Port Erin was measured and it was found that it gave a blue with litmus, orange with neutral red and faint pink with alizarine showing a hydrogen ion concentration of about 1×10^{-8} N.

As the reaction of the tissues is complicated by their relative staining power the organs were usually placed in dilute acid or alkali as the case might be to show that the indicator was present although not visible at first. This was of course necessary with such dyes as neutral red because in this case the yellow colour of the alkaline reaction was not visible but the red acid state could be easily distinguished.

Cœlenterata.

(*Actinoloba dianthus.*)

Whilst collecting material for this portion of the work an unusual specimen was discovered which consisted of two anemones joined together by a common base (15 cms. long). It is probable that this was a case of partial fission as both were of practically the same size[13]. In order to determine if there was any connection between the two body cavities, one mouth was supplied with food stained by neutral red. The tentacles of that disc became pink whilst those of the other remained uncoloured for the four weeks that it was kept under observation. Stimulation of the tentacles of one disc caused a retraction of both portions but the unstimulated side was somewhat later in responding. It is probable that the latter was stimulated to contract by the pull of the contraction of the first half.

One of the first experiments was to determine whether anemones could absorb and retain dyes. A piece of fibrin was stained with methylene blue and an anemone fed with it. Within eighteen hours the anemone became blue and the colour gradually increased for a day. The staining was remarkably persistent, as the anemone remained for over seven months in the aquarium tanks, and is still alive, with the blue colour retained. Mr H. C. Chadwick, the curator of the Biological Station, informs me (Dec. 1909) that the colour has recently faded in places so that the blue is now confined to bands running circularly round the column.

It was usually fairly easy to feed the anemones. Fibrin, Patella foot and Pecten adductor were all taken. In some cases however the anemone could be seen to expand and push the food away with its tentacles until it was removed from the surface of the disc. The behaviour of anemones and their discrimination of food is described by Parker[12] and therefore need not be considered further.

When food is swallowed it is usually rapidly disseminated throughout the mesenteric filaments; and, from the detailed experiments of Jordan[6], this is evidently due to the action of an enzyme. In one case where boiled fibrin stained with congo red had been used, the fibrin was not digested but remained as a solid mass at the lower end of the œsophagus. Krukenberg found that boiled fibrin was not digested by astacus[9].

The results of feeding anemones are summarised as follows:

Congo red. Tentacles and column become pink. The central portion of the disc appeared mauve but this effect may be purely optical. The colour faded in about three weeks.

Vesuvian brown. No colour visible.

Sodium alizarine sulphonate and *gallein.* Cause a faint pink colour which disappears within a week.

Rosolic Acid. Not absorbed, red mass regurgitated as if fibrin had been digested and the rosolic acid left as an insoluble residue.

Litmus and *Lacmoid.* No definite colour.

Aniline blue. Tentacles and mouth marked blue, intervening portion of disc paler in colour.

Neutral red. Tentacles pink, mesenteric filaments deep red.

Alizarine. No definite colour but mesenteric filaments become pink with alkali.

These experiments leave much still to be desired, but the mesenteric filaments were evidently on the acid side of neutral red and alizarine. Further experiment is necessary using indicators which show better colour changes before the question as to the actual hydrogen ion concentration of the anemones can be definitely settled.

Echinodermata.

(*Echinus esculentus.*)

The sea urchin was found to eat fibrin, Patella foot and Pecten adductor. It is usually stated that the food of sea urchins is sea-weed [18]. Scott [18] on the New Brunswick coast found the intestine usually full either of sea-weed or surface sand; whilst Chadwick [1] states that the sea urchin is wholly carnivorous. In most of the specimens collected from the ruined break-water at Port Erin, which is covered with barnacles (*Balanus*) and sea-weeds, there were sharp angular pieces of calcareous shell, such as would be obtained by crushing barnacles, and no sea-weed in the intestine; but in some cases there was sea-weed and occasionally this was present in large amount.

Some pieces of rock, covered with barnacles, were placed in the tank containing the sea urchins. In a short time several sea urchins were seen on the surface of these stones and later a number of the barnacle shells were found to be empty with the margins of their shells broken. One of these pieces of stone, about forty cubic centimetres in

volume, was carried forty-one centimetres up the glass face of the tank by an Echinus, and a small sea urchin raised a pecten shell, with a large barnacle on its under surface, so that it could reach the barnacle. It would therefore appear that the sea urchin eats both animal and vegetable food.

Through the glass of the aquarium tank, the manner of feeding and the actions of the pedicellariæ, spines, tube feet and mouth were observed, as follows:

Pedicellariæ. When food is dropped on the surface of an Echinus, the pedicellariæ suddenly become on the alert, but they do not appear to attach themselves to immobile food. Experiments were tried by placing specimens of the worm *Nereis fucata* on Echini. The worms were seized by the pedicellaria and were conveyed towards the mouth. The functions of the pedicellaria have, however, been fully described by v. Uexkull [21], so it is not necessary to go into further details.

Spines. As soon as the food comes into contact with the Echinus the spines begin to move slowly and deliberately. They act by pushing the food in the required direction. It is extremely interesting to watch how all the spines seem to be acting independently yet they are all integrated to produce a common result. The spines behind push the food whilst those before bend down, sweep to the side and attempt to pass the margin of the food so as to get behind it and push in their turn. If they do not bend far enough to the side but come into contact with the food they extend farther to the side and repeat the manœuvre until they can pass without touching. When the food reaches the mouth the spines surrounding the peristome all bend inwards to prevent the food from being lost. Many interesting points in the response of the spines to stimulation have been described by v. Uexkull [22].

Tube feet (*Podia*). At the same time that the spines are moving the food the tube feet extend in the direction of the food and some of them become attached thus helping to pull the food towards the mouth. Sometimes an Echinus can be seen with a number of tube feet lying parallel and close together along the ambulacral area, in the direction of the aboral pole.

The part played by these organs is somewhat variable. With dead food the pedicellariæ do not appear to act, but in the experiments with Nereis the pedicellariæ were of great importance in holding the worm until the spines could force it towards the mouth. Sometimes the spines seemed to act alone but usually they directed the food towards the ambulacral area and then the tube feet could act more advantageously.

The food was sometimes moved quite rapidly and at others slowly as if the animal did not require it urgently. The rate was not dependent on temperature as far as one could judge. If the food was not to be eaten the movement was away from the mouth mainly by the action of the spines but in some cases the tube feet seemed to assist by pushing the food away. When the rejected food was carried to a suitable position it was allowed to drop off the shell to the bottom of the tank.

Mouth. If the food was to be eaten, rhythmical movements of the mouth commenced soon after the edible matter was placed on the Echinus and long before it reached the vicinity of the mouth. The movements were at first slight and gradually became more marked. The mouth was directed towards the side from which the food was approaching. The mouth opens widely exposing the œsophageal folds, then the teeth are thrust out, brought together and withdrawn. As the teeth are withdrawn the lips are thrust out in a funnel shape and the mouth returns to its central position. During half the time of each movement the teeth are closed and give one the appearance of crushing or grinding anything which might be between them. During one of these movements when the teeth are projected towards the side from which the food is coming, a portion of the food is seized between the teeth. As the teeth are withdrawn and the lips protruded the food is partially engulfed and at the same time drawn towards the centre of the peristome. The mouth movements sometimes occurred although the food was rejected.

The rapidity of the mouth movements was found to vary with the temperature so a few observations were made to calculate the temperature coefficient. The movements were counted for some minutes and the rate per minute determined.

Effect of Temperature on Rate of Movements of Mouth of Echinus esculentus.

Observation	Temperature of tank	No. of movements of mouth per min.	Observations	Temperature difference	Temperature coefficient
1	7·2° C.	1·0	1—2	9·4° C.	3·2
2	16·6° C.	3·0	1—3	7·5° C.	3·2
3	14·7° C.	2·4	1—4	6·7° C.	3·5*
4	13·9° C.	2·3	2—3	1·9° C.	3·2
			2—4	2·7° C.	2·6*
			3—4	0·8° C.	1·6*

* Observation 4 is apparently at fault. If the number of observed movements per minute had been 2·2 the divergent coefficients would have been 3·3, 3·1 and 3·0 respectively, which would make the table uniform.

In all of these determinations except the last, where the temperature difference is very small, the coefficient is evidently one depending on a chemical reaction. In a review by Snyder[18], where a collection of temperature coefficients has been made, there does not seem to be any case analogous to this where a rhythmical reflex shows a temperature coefficient independent of the conditions of stimulation; that is, in this case, the desire of the animal for food.

The food in the intestine was found in small round masses usually about 2—4 millimetres in diameter. Scott[19] states that the food is usually surrounded by mucus but owing to the nature of the food (seaweeds) he could not observe the points to be given here. In the first part of the intestine the food was solid and opaque and when indicators were present showed colours. Sometimes there was seen to be a slight mucus-like layer surrounding the food particles but usually they only appeared smooth and polished with all the inequalities rounded off. Lower down the intestine the particles become transparent. The opaque mass gradually diminishes from the surface inwards leaving a transparent border. At certain stages these particles appear with a solid core surrounded by a transparent jelly-like layer. Finally there is left a transparent spherical mass sometimes with coloured contents or a coloured precipitate. Surrounding the spherules there was a distinct membrane, which was fairly tough as it showed some resistance to rupture. When ruptured the contents escaped and the membrane was left as a slightly opaque bag which was not soluble in either dilute acid or alkali.

Variations from this typical condition were only occasionally seen. In one specimen the food was not so thoroughly masticated, as twenty-four hours after feeding, a large torpedo-like mass over one centimetre long was found at the top of the œsophagus.

After finding that the digestion occurred in a capsule not unlike the description of a food vacuole in infusoria, the contents of the intestines of freshly collected Echini were examined. The masses of broken barnacle shells were found to be held together by a similar membrane and the excreta deposited in the tank, after fresh Echini had been put in, were also collected in small spheres surrounded by a membrane.

During the course of the experiments it was noticed that whenever the intestine was touched a slow contraction of the intestinal muscles resulted such as usually occurs in mammalian involuntary muscle.

From a large number of experiments on the digestion of sea urchins by the method of indicators the following results are selected. Usually

at the end of twenty-four hours the food seemed collected at the junction of the superior and inferior spirals of the intestine. The food could be found at the junction of œsophagus and inferior spiral in about four hours. Comparatively rarely the food was found in intermediate portions but the food was traced all through the intestine and the colours noted. Even before the food reaches the apex of the test in the œsophagus it is seen to be rounded and smooth on the surface as if already surrounded by a capsule or mucus.

The food was dyed or impregnated with indicators. At varying intervals the animals were opened and the round masses in the intestine examined. After noting the colour the food particles were placed in acid or alkali to see if any colour change resulted.

At the end of 24 hours:

Di-methyl-amido-azobenzol	Yellow becoming red with acid.
Congo red	Red becoming blue with acid.
Vesuvian brown	Yellow turning brown with acid.
Sodium alizarine sulphonate	Purple decolourised by acid.
Gallein	Purple decolourised by acid.
Rosolic acid	Pink decolourised by acid.
Litmus	Blue becoming pink with acid.
Neutral red	Orange becoming red with acid and yellow with alkali.
Alizarine	Some masses purple becoming colourless with acid.
Phenolphthalein	Colourless becoming pink with alkali.

These results showed a reaction, approximately that at which neutral red changes colour, so most of the experiments were performed with litmus, neutral red and alizarine.

Neutral red stained food becomes yellow in sea water. Soon after it is eaten (about four hours) it becomes red, but the food masses do not become acid to litmus. Lower down the intestine the neutral red seems to leave the food as in most cases no red colour was produced even in acid; however, a number were found with yellow masses which became red in acid. This would seem to indicate that the food becomes relatively acid in the first portion of the intestine and then returns to the alkaline colour at the same time the indicator diffuses out of the food masses.

Starfish (Asterias rubens).

The behaviour of starfish has been described by Jennings[3] who has observed their manner of catching food and conveying it to the mouth. The starfish were more fastidious than anemones or sea urchins as under no conditions would they eat fibrin, but in most cases

they could be persuaded to take *Pecten* adductor or *Patella* foot. When food was rejected it was actively pushed away by the tube feet. It is interesting to compare this action with the walking movements as described by Jennings. He says that they walk by pushing with their tube feet and not pulling, since they walk as well on sand as on smooth hard surfaces, and that in walking up a vertical surface they must adhere by their suckers. However it appears that even under these conditions they also push as on watching them climb up the glass surface of the aquarium tanks the tube feet can be seen to extend backwards and when the sucker is released the foot jerks off as if released from a spring. After which the foot coils up and extends forward for the next step.

Most of the feeding experiments were performed with congo red, and the following is an abstract of a typical experiment on a series of animals fed on *Patella* foot stained with congo red.

One and a half hours after feeding, stomach protruded surrounding food and the latter partially projecting into the starfish. Pyloric cæca yellowish brown, duct up centre faintly red coloured. Rectal cæca uncoloured.

Three and a half hours. Food swallowed although stomach is still everted. Food contained in stomach showed a doubtful purple tinge on the surface becoming a brighter red with alkali. Pyloric cæca purplish turning pink with alkali. Rectal cæca faint blue becoming faint pink with alkali.

Six and a half hours. No sign of food but stomach becomes everted when the animal is handled. Food still in stomach but outside layers have obviously been removed. Pyloric cæca dark brown with a bluish shade becoming red with alkali. Rectal cæca blue but on placing in alkali they contract and squeeze out a fluid which is instantaneously turned red.

Ten and a half hours, very much like the preceding. Food nearly all digested.

Fourteen hours. Pyloric cæca dark brown with a bluish shade, ducts show a distinct blue as if containing a blue solution. Rectal cæca distinct blue. These all became red in alkali.

Thirty-seven hours, as preceding observation.

Sixty hours. No food in stomach, pyloric cæca dark blue brown rectal cæca pink.

Di-methyl-amido-azobenzol did not cause any definite colour even when the glands were treated with acid.

Vesuvian brown showed brown colour becoming lighter with alkali. As far as one could judge the acidity was not so marked in the summer experiments as in those carried out at Easter.

Porania pulvillus (*in summer*).

With congo red the pyloric cæca showed a russet colour becoming light red with alkali and blue with acid.

During digestion of food stained with vesuvian brown, the stomach being everted it was distinctly noticed that the margin of the food in contact with the stomach wall was dark brown in colour whilst the rest of the food was yellow.

There is very little doubt that during digestion the reaction is approximately that of a decimolecular solution of sodium di-hydrogen phosphate (H^+ about 1×10^{-4} N). After digestion of congo red stained food had finished, the rectal cæca became pink but it was not determined how alkaline the contents became. The pyloric cæca remained a brown colour even after all the food was digested.

MOLLUSCA.

(*Pecten opercularis.*)

After many attempts this was the only form of mollusc which could be induced to take the dyed food. At Easter time sea urchin eggs were used, and in the summer, Patella eggs. With some indicators the eggs would not stain or when stained the colour diffused into the sea water. Congo red seems to be the most suitable stain except that it requires a relatively strong acid to cause its colour to be changed. The only other indicator which could be found in the intestinal tract after feeding was vesuvian brown.

The eggs were obtained by triturating the ovaries in fresh water and a solution of congo red in freshwater was added. After some hours the egg mixture was poured into a large flat dish containing the specimens of *Pecten opercularis.*

The chief interest of these experiments is in relation to the crystalline style. In Dakin's L.M.B.C. Memoir on *Pecten*[8] he states that it disappears on starvation and reappears when food is given. It contains an enzyme that can digest starch and the spiral and other markings on the same green coloured material as is found in the stomach.

Johnstone[m] believes that the style is associated with the ingestion of a large amount of foreign matter with the food and that it is the first separation of the food from the indigestible constituents.

Owing to the style becoming a purplish pink colour when placed in dilute acid, indicators which became red in acid could not be traced. In alkali the style dissolves so indicators which became coloured by alkali were also inapplicable.

The food particles are so small that it is difficult to trace their fate especially as the amount of indicator contained in the eggs is very small.

Congo red eggs caused stomach contents which were russet brown in colour. In some of the specimens the styles showed either a core or spiral markings of reddish granular material which on treating with acid became blue. In one of these specimens there were spiral markings which were formed of spirals of different diameter, so that on placing in acid a series of concentric blue spirals appeared against the purplish red colour of the normal style when treated with acid. In another instance a well marked brown figure-of-eight mass was seen in the stomach end of the style and this became blue with acid.

Vesuvian brown gave brown stomach contents and brown markings in the style.

As these experiments seemed to indicate that food materials pass into the style, some specimens of *Pecten* were fed on diatoms. Owing to there being no sea water diatoms available during August an emulsion of fresh water diatoms was added to sea water in which the *Pecten* were kept. After about two days the *Pecten* were opened and the styles kept in seventy per cent. alcohol. Free hand sections of the styles were made and examined. Diatoms were found imbedded in the styles in some places showing a spiral arrangement. Mitra[n] also found food particles inside the styles.

Certain experiments were made by placing *Pecten* in sea water containing indicators in solution. The two tissues which were mostly stained by the indicators were the gills and kidneys.

Vesuvian brown stained the gills brown and the kidneys yellow, the latter becoming brown in acid.

Sodium alizarine sulphonate, gills pale yellow, kidneys dark red.

Alizarine, gills pale yellow, kidneys yellow becoming crimson with alkali.

With these indicators the gills were always relatively acid to the kidneys but the differences in colour might have been due to differences in absorption of the dyes. Neutral red gave more definite results.

Neutral red, gills bright red, whilst the testes were pink and the kidneys were yellow. On placing portions of the testes and kidneys in dilute acid they became bright red thus showing that the indicator was present although in the kidneys no red colour was visible until treated with acid.

It would appear from these experiments that probably the different organs of an animal may possess different hydrogen ion concentrations.

Dakin[2] states that *Pecten opercularis* rarely except when small attaches itself by a byssus but the specimens kept in a large tank showed a tendency to become attached by byssus threads. One was actually seen with the foot out secreting and attaching the threads. It may be that owing to the continuous circulation through the tanks there was no need for the *Pecten* to move in search of food and hence they showed a greater tendency to attach themselves. The transverse measurements of the shells of those found attached were 5·2, 5·2 (two threads), 5·3, 5·3 (seven threads), 5·4, 5·4, 5·8 (three threads), 6·6, 7·0 (one thread) and 8·0 (seven or eight threads) centimetres respectively.

Summary and Conclusions.

During the course of experiments made to investigate the presence of acid or alkali, during digestion, in the intestine of marine invertebrates, observations were made on the habits of the animals studied. In comparing the results it is well to point out that the true neutral point corresponds to hydrogen ion concentration of about 1×10^{-7} N. Any value greater than this denotes acidity, any smaller value shows alkalinity.

Actinoloba dianthus. A case of partial fission was investigated and it was found that the two individuals although structurally united were physiologically independent.

The reaction of the tentacles is practically neutral since they become faintly red after the animal is given neutral red stained food ($H^+ > 1 \times 10^{-8}$ N). The mesenteric filaments showed a deeper red, pointing either to a more acid condition or a greater concentration of the dye.

Echinus esculentus. The food of *Echini* from the neighbourhood of Port Erin is chiefly composed of barnacles but sea-weed is also found in the intestine.

During feeding the pedicellariæ, spines and tube feet all participate in conveying the food to the mouth.

The rhythmical mouth movements were found to have a temperature coefficient of about 3·2.

The food in the intestine exists in the form of oval masses surrounded by a capsule, as digestion proceeds the opaque contents of these capsules become transparent.

During digestion the contents of the food capsules were found at first to become relatively acid ($H^+ > 1\times10^{-6}$N) and then they became more alkaline ($H^+ < 1\times10^{-6}$ N).

Asterias rubens. During digestion the pyloric cæca become acid to congo red ($H^+ = 1\times10^{-4}$ N). The rectal cæca also become filled with a liquid of the same acidity but in the later stages the acidity of their contents diminishes.

Porania pulvillus. The pyloric cæca of this species also become acid to congo red during digestion.

Pecten opercularis. The crystalline style becomes coloured by food stained with congo red or vesuvian brown. After the *Pecten* are fed on diatoms the skeletons of the latter are found inside the style. The crystalline style is probably formed by the mixing of the food with the secretion of the digestive gland.

By keeping *Pecten* in solutions of indicators the gills show a more acid reaction than the kidneys. This is of importance as it suggests that tissues do not all have the same concentration of hydrogen and hydroxyl ions.

Measurements of adult *Pecten* which became attached by byssus threads are recorded.

In regard to the evolution of enzymes and the reaction in which they act, the experiments show the following results:—

Starfish show definite acidity.

Pecten probably possess acidity. The indicators were not sufficiently concentrated in the crystalline style to show the reaction clearly but the stomach contents were russet brown after feeding with congo red stained eggs (H^+ probably about 1×10^{-4} N).

In sea urchins there was alkalinity during the greater portion of the time but in the early stages the reaction became neutral or faintly acid. These results point to a condition similar to that found in protozoa, namely that during the first stage digestion occurs in the presence of acidity and later in the presence of alkalinity. It is probable that digestion takes place by one enzyme that has not yet become so differentiated as to be able to attack proteins in one reaction only.

Anemones show a neutral or faintly acid reaction.

Neutral red, gills bright red, whilst the testes were pink and the kidneys were yellow. On placing portions of the testes and kidneys in dilute acid they became bright red thus showing that the indicator was present although in the kidneys no red colour was visible until treated with acid.

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Anemones show a neutral or faintly acid reaction.

In conclusion I wish to thank Professor W. A. Herdman and Mr H. C. Chadwick for valuable help and advice during the course of these investigations.

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THE OXYGEN CAPACITY OF THE BLOOD AFTER HÆMORRHAGE.

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Haldane and Lorrain Smith[1], Barcroft and Morawitz[2] have shown that under normal circumstances the oxygen capacity of the blood runs parallel to its depth of colour as judged by the hæmoglobinometer. Morawitz and Röhmer[3] have recently shown that this statement also holds good for man in various conditions of anæmia: amongst other pathological states they examined cases of chlorosis, pernicious anæmia, post-hæmorrhagic anæmia, and anæmia occurring in malignant disease.

It seemed of importance to examine this point in cases of post-hæmorrhagic anæmia in somewhat greater detail than did the last two observers, in view of the fact that Bohr has shown that the relation of oxygen to the iron in the blood or hæmoglobin is by no means constant in different individuals of the same species[4], and is liable to marked alteration in the same individual under various circumstances, for instance after hæmorrhage[5]. This fact has led him to suggest the occurrence of more than one hæmoglobin. In dealing with post-hæmorrhagic anæmia from the present point of view one hopes that if different hæmoglobins do in reality exist, such will reveal themselves at a time when the animal is rapidly regenerating blood to make good a serious loss.

[1] Haldane and Lorrain Smith. This *Journal*, xxv. p. 335. 1900. Haldane, *Ibid.* xxvi. p. 497. 1901.

[2] Barcroft and Morawitz. *Deutsch. Arch. f. klin. Med.* xciii. p. 223. 1908. *Proc. Physiol. Soc.* p. lvi. 1908. (This *Journal*, xxxvi.)

[3] Morawitz and Röhmer. *Deutsch. Arch. f. klin. Med.* xciv. p. 529. 1908.

[4] For a discussion of this aspect of the question cf. Bohr's articles on the blood gases in Nagel's *Hdb. d. Physiol.* i. p. 93.

[5] Bohr. *Bull. d. l'Acad. Roy. Danoise des Sci. et des Lettres.* 1890. Tobiesen, *Ibid.* 1895.

Rabbits were the subjects of all the experiments described below, and were bled to the required degree by puncturing a vein in the ear with a surgical needle, no anæsthetic being required. The hæmoglobinometer employed was Haldane's[1].

Before determining the maximal oxygen capacity by blood gas analysis the blood was thoroughly oxygenated by shaking with air in a large flask at room temperature.

At the outset the original ferricyanide method of Haldane[2], which necessitates 20 c.c. of blood for each determination, was used for determining the blood gases. It was however found impossible to obtain constant readings[3] with this instrument when dealing with rabbit's blood, especially in the later stages of hæmorrhage: the volume of gas under measurement slowly and continuously diminished, presumably from the absorption of oxygen. On the hypothesis that it might be due to the abnormal presence of easily oxidisable substances in the anæmic blood indicative of deficient oxidation in the tissues, the blood was kept warm after withdrawal from the animal, rapidly aerated and then incubated at 37° for so long as an hour. It was then cooled down to room temperature, aerated again and its gases determined. This procedure was however without effect on the subsequent diminution of gas volume in the apparatus. Further enquiry showed that this phenomenon did not occur, or, if it did, to only a very slight extent, until ferricyanide was added to the laked blood. The effect of the slow diminution of volume was to give a false impression that the oxygen capacity of the blood was less than would be estimated from the reading of the hæmoglobinometer.

About this time Morawitz and Pratt[4] drew attention to the remarkable absorption of oxygen and production of carbonic acid which occurs in the blood of rabbits made anæmic either by the repeated injection of phenyl hydrazine or by repeated hæmorrhage. It was therefore evident that the time factor was of importance in making the blood gas determination. The Barcroft-Haldane apparatus[5] was consequently substituted for the original form, since owing to the small amount of fluid dealt with in it the delay in assuming the temperature

[1] Haldane. This *Journal*, XXVI. p. 497. 1901.

[2] Haldane. *Ibid.* XXV. p. 295. 1906.

[3] This fact has been noticed by others who have employed the ferricyanide method. Cf. Morawitz, *l.c.* p. 311. Plesch. *Zeitschr. f. exp. Path. u. Therap.* VI. p. 474. 1909.

[4] Morawitz and Pratt. *Münch. med. Wochenschr.* LV. p. 1817. 1908. Morawitz. *Arch. f. exp. Path. u. Pharm.* LX. p. 298. 1909.

[5] Barcroft and Haldane. This *Journal*, XXVIII. p. 232. 1902.

TABLE I.

Number and weight, in kilos.	Date	Cc. of blood removed	% of haemoglobin in blood of animal. [Haldane scale]	Cc O_2 capacity % of blood by			% of oxygen disappearing from the blood during incubation at 37°	Coagulation prevented by
				Ferricyanide method	Haemoglobinometer	Haemoglobinometer value in % of ferricyanide value		
1. 4·1	Sept. 13	2·9	83	13·3	13·1	98·5	—	Oxalate
	15	3·2	82	13·05	13·1	100·4	—	,,
	16	3·6	81	13·45	13·2	98·2	—	,,
	17	4·0	85·5	14·3	14·2	99·3	—	,,
	18	29·0	75	12·05	11·9	99·2	—	,,
	23	3·9	75·5	12·45	12·5	100·4	—	,,
	24	33·0	68·5	11·4	11·1	97·4	—	,,
	28	56·0	70	12·4	11·9	96·0	—	,,
	29	4·3	55	9·1	9·2	101·0	—	,,
	30	5·5	58	9·85	9·9	100·5	—	,,
2. 5·5	Sept. 11	2·8	81	12·6	12·8	101·5	—	Oxalate
	15	3·0	87·5	14·1	14·0	99·3	—	,,
	28	40·0	82	13·9	13·6	97·9	—	,,
	29	4·4	72·5	12·45	12·1	97·2	—	,,
	30	4·4	71	12·15	11·85	97·5	—	,,
	Oct. 5	50·0	71	13·15	12·9	98·0	—	,,
	7	51·0	67	11·85	11·4	96·2	—	,,
	11	44·0	70	12·0	11·75	98·0	7·1 % in 1 hr. 20 mins.	,,
	12	47·0	63	10·6	10·6	100·0	6·8 ,, 1 ,, 40 ,,	,,
	13	5·0	56·5				37·0 ,, 1 ,, 0 ,,	Citrate
	14	7·8	58	9·5	9·55	100·5	—	Oxalate
	16	5·7	65	11·35	11·1	97·8	—	,,
3. 4·1	Oct. 18	6·0	78	13·5	13·4	99·3	3·7 % in 0 hr. 30 mins.	Citrate
	20	45·5	75·5	13·2	13·0	98·5	4·1 ,, 1 ,, 30 ,,	Fluoride
				12·3	12·3	100·0	9·3 ,, 1 ,, 0 ,,	Citrate
	22	17·5	58·5	10·1	10·3	102·0	—	Fluoride
				9·9	10·1	102·0	11·4 % in 1 hr. 0 mins.	Citrate
	25	47·0	59·5	10·5	10·3	98·1	6·7 ,, 0 ,, 50 ,,	Fluoride
							25·0 ,, 0 ,, 50 ,,	Citrate
	27	41·5	54	9·7	9·35	96·4	74·0 ,, 2 ,, 0 ,,	,,
							25·5 ,, 2 ,, 0 ,,	Fluoride
	29	14·5	53	10·4	10·2	98·1	52·4 ,, 2 ,, 0 ,,	Citrate*
	Nov. 1	10·5	67	12·6	12·3	97·7	30·5 ,, 2 ,, 0 ,,	Citrate*
4. 4·3	Nov. 3	10·0	68	11·95	11·5	96·2	21·0 % in 2 hrs. 0 mins.	Citrate
	8	11·5	71	13·0	12·2	93·8	16·6 ,, 2 ,, 0 ,,	,,
	10	54·0	64·5	11·6	11·2	96·6	22·4 ,, 2 ,, 15 ,,	,,
	12	51·0	52·5	9·0	9·1	101·1	84·0 ,, 3 ,, 0 ,,	,,
	13			9·4†			—	,,
	15	40·0	51·5	8·65			19·4 % in 0 hrs. 30 mins.	,,
	17	39·0	50·5	9·4	9·0	95·7	96·5 ,, 2 ,, 15 ,,	,,
	25	18·5	69·5	12·75	12·3	96·5	—	,,

* Washed corpuscles suspended in Ringer's fluid.

† Blood of Nov. 12, re-aerated after keeping for 20 hours at 6°.

of the water-bath is greatly reduced, and moreover the time required for the complete evolution of the gases is very short. This method has, too, the advantage that a definite reading can be obtained before the slow diminution of volume described above manifests itself.

The values which were obtained in this way are given in Table I[1]. Table II gives similar values which were obtained with normal untreated animals.

TABLE II.

Number	Cc. O_2 capacity per 100 c.c. of blood by		Hæmoglobinometer value in % of ferricyanide value
	Ferricyanide method	Hæmoglobinometer	
1	13·3	13·1	98·5
2	12·6	12·8	101·5
3	13·5	13·4	99·3
4	11·95	11·5	96·2
5	11·4	11·4	100·0
6	15·2	14·65	96·4
7	13·55	12·95	95·6
8	12·2	12·1	99·2
9	11·95	11·9	99·6
Mean	12·85	12·65	98·5

In each case the oxygen capacity calculated from the reading of the Haldane hæmoglobinometer shows but a slight variability from that actually determined by the ferricyanide method. This variability does not appear to be altered in any definite direction as a result of the hæmorrhage, nor does it after the bleeding extend over a wider range than that which was found in the normal animals. From Table II it will be seen that the hæmoglobinometer gave values which are on the average 1·5 % too low in the case of rabbit's blood, with a variation up to ±3 % of the mean. Such variations were somewhat greater than were found for ox or human blood (the hæmoglobinometer employed gave readings in this case which were 1 % too low, and all the values given in this paper have been corrected for this error). The discrepancy in the case of the rabbit's blood can be explained in part at least by the fact that its tint when saturated with carbon monoxide is somewhat yellower than the standard which is made from ox blood, for there is a tendency to underestimate the depth of tint of the solution if that

[1] It should be noted that the fourth column of Table I refers to the blood in the animal, whilst the fifth and sixth columns refer to blood which was diluted with the anticoagulant.

solution has a yellower tint than the full pink of the standard. The tendency to yellowness is more marked in some animals than in others; animal 4 was particularly conspicuous in this respect.

In the experiments detailed above there is nothing resembling the large variations of oxygen capacity which have been found by those who have adopted the iron as their standard.

It is true that somewhat similar variations to the last have been noted in some cases by those who have employed the spectrophotometric method of estimating the concentration of the hæmoglobin, but in this instance there does not seem to be sufficient evidence to show that these were not due to the errors incidental to the method.

Taking the present series of experiments in conjunction with those of the observers whose names have been mentioned at the commencement of this paper, I would suggest that, so far as the animal is concerned, the important relation is that of the oxygen to the hæmoglobin as such, and that there is but one type of hæmoglobin. It may be that the inconstancy of the ratio of the oxygen capacity to the iron indicates that there is a variable quantity of iron in the blood or in the hæmoglobin as usually prepared, which is not concerned in the transference of oxygen, which is not in other words an integral part of the hæmatin moiety of the hæmoglobin molecule.

It has been already shown by Barcroft, Camis, and King[1] that the variations in the dissociation curves of different kinds of blood are due not to the hæmoglobin but to the accompanying salts and to the temperature. There is thus no remaining valid argument against the identity of different varieties of hæmoglobin.

In some of the experiments described above the oxalate, fluoride or citrate blood was after thorough aeration at room temperature incubated at 37°, blood gas analysis being made both before and after this incubation. The rate of disappearance of oxygen from the blood was of the same order as that found by Morawitz, and the actual values obtained are noted in the eighth column of Table I. That neither the venous character of the blood nor the presence of the salt is material to the oxidation is shown by Morawitz's experiments, since he employed defibrinated blood obtained from the carotid artery. It will be seen that the oxidation process increases with repetition of the hæmorrhage, that is at a time when the red cells and hæmoglobin are being regenerated most actively. This activity is evidenced not only by the

[1] Barcroft and Camis. This *Journal*, XXXIX. p. 118. 1909. Barcroft and King. *Ibid.* XXXIX. p. 374. 1909.

course taken by the hæmoglobin percentage, for notwithstanding the repeated bleeding this value did not fall below 50 %, but also by the progressive polychromasia shown by the red cells together with the occurrence of nucleated red cells.

The presence of fluoride slowed the absorption considerably but did not stop it, and the same may be said of oxalate. In the last case no actual blood gas analyses were made in order to compare the oxidation in oxalate and citrate blood derived from the same source: it was however quite evident that the citrate blood darkened on incubation much more rapidly than the oxalate blood.

In one experiment the oxidation process was followed in greater detail by making both oxygen and carbonic acid estimations at various intervals after the commencement of incubation. The result was as follows (Table III).

TABLE III.

	Oxygen %	CO_2 %
Directly after aeration	8·7	30·8
After incubation for ½ hour	6·0	34·8
" " 1½ hours	2·8	36·0
" " 2½ "	0·3	39·1
" " 3½ "	−1·3	39·9

The figures show that practically the whole of the oxygen had disappeared from the citrate blood after 2½ hours with a corresponding development of carbonic acid. After 3½ hours a slightly negative value —an actual absorption of oxygen from the air in the blood gas apparatus presumably—was obtained for the oxygen with a slight increase in the amount of carbonic acid. The respiratory quotient for the whole period works out to 0·91. The same blood when aerated at room temperature contained 9·23 % of oxygen, and blood which was re-aerated again in the same way after it had been incubated for 3½ hours contained 9·48 % of oxygen. The low value for the oxygen given in Table III can be explained by the fact that in this instance the blood was saturated with air at a temperature of 37°, and that there was some delay during which a slight absorption of oxygen might take place before the blood gas determination could be made.

Morawitz concluded as the result of his various experiments (such for instance as working with washed corpuscles, adding serum of anæmic to the blood of normal animals, and examining the nature of the cells in blood which showed a rapid disappearance of oxygen) that the medium

concerned in the oxidative process was cellular, and in the main an expression of the metabolism of young though not necessarily nucleated red cells. The figures given in the first table for experiments made on Oct. 29th and Nov. 1st (animal 3) bear out this statement so far as the corpuscles in general are concerned. On one or two occasions plasma was obtained by centrifugalisation from blood which showed active oxidation and a trace of laked human blood was added to it. Incubation resulted in but little change in this hæmoglobin: on one occasion certainly some of the oxyhæmoglobin was reduced but there was still a considerable amount of oxyhæmoglobin present, even after two hours' incubation. The rate of oxidation in the plasma cannot therefore have been very great.

The shed blood of normal untreated rabbits showed under the same conditions of incubation[1] a distinct disappearance of oxygen and increase of carbonic acid, but at a far slower rate than that derived from the anæmic animals. Table IV shows the figures which were obtained.

TABLE IV.

	Blood gases % of blood before incubation		Blood gases % of blood after incubation at 37°		Respiratory quotient	Period of incubation		
No.	O_2	CO_2	O_2	CO_2		hrs.	mins.	
3	13·5	—	13·0	—	—	0	30	Citrated blood.
4	11·95	—	9·55	—	—	2	0	,, ,,
5	15·35	33·1	13·3	35·5	1·17	1	30	,, ,,
6	12·1	41·7	8·85	44·75	0·94	2	10	,, ,,
			7·35	46·2	0·95	4	20	,, ,,
	12·2	16·45	10·5	17·35	0·53	2	10	Part of plasma removed and replaced by distilled water, corpuscles partly laked.
	12·3	—	11·1	—	—	1	30	Citrated blood kept for 18½ hrs. at 5°.
6 *a**	8·5	19·85	6·9	21·3	0·91	2	0	Part of plasma removed and replaced by twice the volume of distilled water. Corpuscles completely laked.
	10·9	25·9	9·1	28·6	1·50	1	30	Citrated blood kept for 24 hrs. at 5°.
5 *a*†	14·3	24·0	12·7	25·0	0·63	2	0	Defibrinated blood.
			12·85	24·7	0·48	2	0	Ditto + sodium citrate crystals.
9	11·3	37·4	9·55	38·7	0·74	4	0	Defibrinated blood.

* This animal had lost 21·5 c.c. of blood two days previously.

† ,, ,, ,, 13·5 c.c. ,, eleven ,,

[1] The blood was manipulated in sterilised vessels.

This slow disappearance of oxygen from normal blood when kept at 37° seems to be no other than that to which Pflüger[1] drew attention in 1867. He apparently noticed two kinds of oxygen disappearance in shed blood, the one almost instantaneous, the other a slower effect which only became visible when the blood was kept at body temperature. The slow type of oxidation has been taken to indicate the metabolism of the leucocytes, but upon this point I have no evidence to offer[2].

Two experiments of the same nature were made with fresh defibrinated ox blood but gave indecisive results. In each instance the percentage of both oxygen and carbonic acid was slightly less in the blood which had been incubated, but the fall was hardly more than could be accounted for by the error of experiment.

Summary.

The parallel relationship of the oxygen capacity of the blood and the depth of its colour as judged by the hæmoglobinometer was not found to be materially disturbed during regeneration following repeated hæmorrhage in the case of four rabbits. This fact supports the theory that there is but one kind of hæmoglobin, or at least that the hæmatin moiety is identical in all kinds of hæmoglobin.

Various experiments are described confirming recent observations on the rapid disappearance of oxygen and output of carbonic acid in shed blood of rabbits made anæmic by hæmorrhage when the blood is kept at body temperature.

[1] Pflüger. *Zentralbl. f. d. med. Wissensch.* pp. 321, 722. 1867. Cf. also Krogh. *Skand. Arch. f. Physiol.* XXIII. p. 193. 1910.

[2] Warburg. *Zsch. f. physiol. Chem.* LIX. p. 112, 1909 brings forward evidence to show that the main factor concerned in this process is the red corpuscles.

QUANTITATIVE RESEARCHES ON THE SUMMATION OF INADEQUATE STIMULI IN MUSCLE AND NERVE, WITH OBSERVATIONS ON THE TIME-FACTOR IN ELECTRIC EXCITATION.

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(*From the Physiological Laboratory, Cambridge.*)

In a number of papers published in this *Journal* during the last few years I have investigated in detail the response of certain excitable tissues to electric currents of varying duration. From these researches it appears that not only different tissues examined under like conditions, but also like tissues examined under different conditions of temperature and of surrounding medium, show well-defined differences in their response to such currents. All tissues examined behave alike in one matter; in every case the weakest current which will excite becomes progressively smaller as the duration of passage is increased up to a certain limit, and beyond that limit the same strength of current is required whatever the duration. It is in the duration of current at which this lowest limit of the exciting current is reached that a clear difference is found to exist between unlike tissues examined under like conditions or between like tissues examined under different conditions.

There is no need to reproduce here the detailed observations on this matter which have already been published[1]. It will be enough to recall the broad facts which have been established. It has been shown that the current-duration at which the lowest limit of the exciting current is first reached is shorter in the motor nerve-fibres to the toad's gastrocnemius than in the sartorius muscle-fibres of the same animal, is shorter in certain tissues of the frog than in the corresponding tissues of the toad, is shorter both in muscle and in nerve when these tissues

[1] References to the original observations made by myself and by Lapicque are given in a later part of this paper (cf. pp. 470, *et seq.*).

are warm than when they are cool, and is shorter in the sartorius muscle of the frog when the concentration of calcium salts in the bathing fluid is high than when it is low.

By another method of investigation I have shown a similar range of differences to exist[1]. For the excitation of any tissue the exciting current must increase from zero with a certain minimum rapidity; and this minimum rapidity or minimum current gradient is steeper for the motor nerve-fibres to the gastrocnemius than for the sartorius muscle-fibres in the toad, is steeper for the nerve-fibres to the gastrocnemius in the frog than in the toad, and is steeper for a muscle surrounded by a solution containing a higher concentration of calcium salts.

The parallel variation of these two phenomena as we pass from tissue to tissue or from one condition to another at once suggests that the existence of a limiting current-duration beyond which the current strength required for excitation no longer falls, and the existence of a limiting steepness of current increase below which no excitation will result, are two expressions of a single property characteristic of each excitable tissue. I have elsewhere[2] called attention to this probability, and have suggested that we may regard the property in question as the need for a completion of the process of excitation with a certain rapidity. Lapicque[3] has noted the same coincidence, and has referred the two phenomena to what he calls the chronological coefficient of excitation[4]. But this amounts in either case to nothing more than a generalized restatement of the experimental facts. It tells us nothing of the conditions within the excitable cell which determine the demand for rapidity, and it gives us no intelligible account of the observed variations in that demand.

In the present paper I wish to describe a series of experiments which show a third property of excitable tissues, namely the summation of inadequate stimuli, to follow a parallel series of modifications as we pass from one tissue to another and from one condition to another in the same tissue. The method used in these experiments has been to determine the greatest time interval at which two stimuli, of a strength less than the threshold strength by a known percentage, may follow one another and just succeed in provoking an excitation.

This interval, which I shall call the "summation interval" for

[1] Keith Lucas. This *Journal*, XXXVI. p. 253. 1907, and XXXVII. p. 459. 1908.
[2] *Ibid.* XXXVII. p. 459. 1908. Spec. p. 476.
[3] Lapicque. *C. R. Soc. de Biol.* LXIV. p. 6. 1908.
[4] *Ibid.* p. 589. 1908.

a given percentage drop below the threshold value of current, is found to be shortened by the same conditions and for the same tissues as have already been shown to present a shorter limiting current-duration and a steeper limiting current gradient. The hypothesis, which may obviously be founded on these experimental facts, that the summation interval is an expression of the same fundamental property which determines the other two phenomena, leads us a considerable step forward towards the understanding of that property. For in determining the time interval at which a second inadequate stimulus following a former will just become adequate we are measuring the rate at which the excitatory disturbance produced by the first stimulus subsides[1].

The hypothesis leads therefore to the conception that the rate of subsidence of the excitatory disturbance is the fundamental property of an excitable tissue which presents itself to us under the three different aspects. The discussion of this hypothesis and of the inferences to which it leads will be continued after the experimental facts have been described.

Method. The fact that two stimuli, of such strength that they fail when applied singly to cause excitation in a tissue, will become effective when following one another at a sufficiently short interval of time has been confirmed by the work of many observers on many different excitable tissues. The experiments of Engelmann[2] on the ureter of the rabbit, of Richet[3] on the muscles of the crab, of Sertoli[4] on the retractor penis of the dog, of Basch[5] and of Engelmann[6] on the cardiac muscle of the frog are typical of the many observations which have been accumulated. Recently Steinach[7] has shown that the same phenomenon is encountered in a great number of tissues ranging widely over the animal and vegetable kingdoms. But these observations afford no ground for a real comparison of the ability of different tissues to sum the effects of successive inadequate stimuli, because they do not give us quantitative data as to both the essential dimensions which we need for such comparison, namely the time interval separating the two

[1] See below, p. 473, for further discussion of this point.

[2] Engelmann. *Arch. f. d. ges. Physiol.* III. p. 282. 1870.

[3] Richet. *Physiol. des muscles et des nerfs.* Paris, 1882.

[4] Sertoli. *Arch. Ital. Biol.* III. p. 88. 1883.

[5] Basch. *Stzber. der k. Akad der Wiss. Wien.* LXXIX. abth. III. p. 87. 1879. *Arch. f.* (*Anat. u.*) *Physiol.* p. 283. 1880.

[6] Engelmann. *Arch. f. d. ges. Physiol.* XXIX. p. 453. 1882.

[7] Steinach. *Arch. f. d. ges. Physiol.* CXXV. p. 239, p. 290 and p. 347. 1908.

successive stimuli, and the percentage by which their strength is below the threshold strength for one stimulus occurring alone. Steinach for example gives precisely the time interval separating his stimuli, but tells us the strength of the stimuli only in terms of the distance between the primary and secondary coils of an induction coil for which no calibration curve is published in his paper.

If we are to arrive at a just comparison of different tissues in this matter there are two courses open to us. We can either fix the time interval separating the two stimuli, and determine by what percentage they may be decreased below the threshold and still cause excitation; or we can fix the percentage by which the stimuli are below the threshold, and then determine the greatest time interval at which they cause excitation. But it is absolutely essential that both the factors should be properly measured. In my experiments I have fixed the percentage by which the stimuli are less than the threshold value for one stimulus occurring alone (usually at 5%) and have determined for each tissue and condition examined the greatest time interval by which such stimuli may be separated if they are just to cause excitation. In this way I obtain for each tissue and condition a characteristic "summation interval."

The technique of obtaining stimuli always less than the threshold value by the same percentage presented more difficulty than I had anticipated. The method finally adopted after several trials was the following:

The exciting currents were obtained from two similar induction coils made without iron cores in the primary. Each coil was first set up with a ballistic galvanometer in its secondary circuit, and the distance between primary and secondary coils was adjusted to give a convenient excursion of the galvanometer at break of the primary circuit. A resistance was then introduced into each primary circuit, of such magnitude as to reduce the galvanometer excursion by 10% or 5% as might be required. In the course of subsequent experiments the threshold stimulus for the tissue under examination was first found on each coil by adjustment of the distance between primary and secondary coils with the resistances shunted. When currents of the required percentage below the threshold were wanted the resistances were unplugged. This method is very rapid in working, and ensures that the drop of the exciting current shall be the same in all observations.

The primary circuits of the two induction coils contained the knock-down keys of the pendulum which I have described in a previous

paper[1]. The interval between the two induction shocks could therefore be regulated by the degree of separation of the two keys. In the experiments described below it will be seen that the interval separating two stimuli is given to the nearest ·0001 sec. The actual determinations were made to the nearest tenth of a degree of separation of the primary keys. Since a tenth of a degree is approximately equal to ·00008 sec. within the range of swing of the pendulum used in these experiments, the accuracy obtained is probably represented nearly enough when the results are given to the nearest ·0001 sec.

The determination of the threshold for each tissue was carried to the nearest 0·5 millimetre coil distance on each coil. Any attempt at closer setting than this leads to longer delay in the making of observations, and does not increase the accuracy of the results. In every case the threshold was determined both before and after the measurement of the summation interval. The following is a typical observation.

Sartorius of frog. In NaCl 0·6 %, $CaCl_2$ 0·1 %. Cathode 3 mm. from pelvic end. Temperature 12·8° C. 1¼ hours after excision.

(i) With resistances in primary plugged.
Threshold for coil A, 201·5 mm.
" " B, 178 "

(ii) After currents from A and B have been reduced 5 % below threshold by unplugging resistances.

A followed by B at	·0019 sec.	Contraction.
" "	·0023 "	None.
" "	·0021 "	None.
" "	·0020 "	None.
" "	·0019 "	Contraction.

(iii) With resistances in primary plugged.
Threshold for coil A, 201·5 mm.
" " B, 178 "

From these observations I take the value of the summation interval to be ·0019 sec. Of course it would happen from time to time that the threshold would alter a little during the determination of the summation time. In cases of this sort I have obtained two values of the summation interval, one of which must be too large, since the tissue became more excitable while the determination was being made, while the other was too small since the excitability became less. For example, in one case the first threshold found for B was 167·5 mm.; a determination of the summation interval gave ·0009 sec.; the threshold for B had

[1] Keith Lucas. This *Journal*, XXXVII. p. 461, Fig. 1, *H* and *J*. 1908.

in the interval risen to 167 mm.; a second determination of the summation interval gave ·0010 sec., and after this the threshold for B had fallen again to 167·5 mm. In this case ·0009 sec. is too small, and ·0010 is too large.

The tissues subjected to experiment were placed, unless the contrary is stated in any case, in my "fluid electrodes," the particular type used being the modification described by Mines[1]. The temperature was read from a thermometer placed inside the electrodes close to the tissue. When necessary for the maintenance or alteration of temperature a water-bath was used, surrounding the whole electrodes.

Determination of summation interval.

1. *The sartorius muscle of the frog.* The values given here are obtained from muscles taken from four different animals. The muscles were in a solution containing 0·6 % NaCl and 0·1 % $CaCl_2$. They had been in this solution for times not less than one hour and not more than two. At such times they would have come very nearly, as some experiments to be described later will show, into equilibrium with the immersing fluid. These experiments will serve for comparison with the motor nerve-fibres to the gastrocnemius and with the ventricle of the frog, in which observations were made under like conditions. The muscles were always excited in the nerve-free pelvic end.

Exp.	Bathing fluid	Time in fluid	Temperature	Percentage below threshold	Summation interval
1	NaCl 0·6 % $CaCl_2$ 0·1	1¾ hrs.	13·0° C.	5	·0011 sec.
2	,,	1¼	12·8	5	·0019
3	,,	1½	13·5	5	·0016
4	,,	1	13·2	10	·0005

There is a fair agreement between the summation intervals found with the stimuli 5 % below the threshold. When this drop is increased to 10 %, the summation interval is, as might be expected, very much shortened.

2. *The motor nerve-fibres to the gastrocnemius of the frog.* In these experiments the contraction of the gastrocnemius muscle was used as an index of the excitation of the nerve-fibres in the sciatic nerve. The experiments were made on three nerves taken from different animals.

[1] Mines. This *Journal*, xxxvii. p. 428, Fig. 12. 1908.

Exp.	Bathing fluid	Time in fluid	Temperature	Percentage below threshold	Summation interval
5	NaCl 0·6 % $CaCl_2$ 0·1	1½ hrs.	12·8° C.	5	·0005 sec.
6	,,	2½	13·7	5	·0004
7	,,	2½	12·3	5	·0005

It appears from these results that the summation interval for the sartorius muscle is about three times as long as that for the nerve-fibres to the gastrocnemius under like conditions.

3. *Ventricular muscle of the frog.* The Stannius ventricle was used. Excitation was by platinum electrodes on the base of the ventricle, since the coreless coils did not give a strong enough current to excite with the fluid electrodes. Owing to the greater difficulty of getting steady results with the heart, and to the relatively great length of the summation interval in this muscle, the determination was made to the nearest 1° of separation of the keys (*i.e.* to the nearest ·0008 sec.), and the times are accordingly given only to the nearest 0·001 sec. This approximation is sufficient where the difference is so large as it is between the summation intervals for skeletal and for cardiac muscle. With the currents 5 % below the threshold the summation interval for the ventricle is five times as long as that for the sartorius. With the currents 10 % below the threshold the corresponding ratio is 6 to 1. The experiments given below were made with different hearts.

Exp.	Bathing fluid	Time in fluid	Temperature	Percentage below threshold	Summation interval
8	NaCl 0·6 % $CaCl_2$ 0·1	1 hr.	13·5° C.	5	·008 sec.
9	,,	1½ hrs.	14·5	10	·003

4. *The effect of calcium salts.* Experiments were made upon this question by two methods. One method was to determine the summation interval in a fluid rich in calcium salts, to substitute a fluid very poor in calcium salts, and after half an hour to make a fresh determination, then to return to the original solution in order to see whether the change observed was really due to the removal of calcium. This was the method used in Exps. 4 and 10 given below. As may be seen from these experiments it was not possible by replacing the fluid rich in calcium to return quite completely to the conditions found before the calcium was removed. But in both experiments the summation times in the fluids rich in calcium are considerably the shorter both before and after the use of the fluid poor in calcium.

Sartorius of frog excited in non-neural region.

Exp.	Percentage below threshold	Temperature	Bathing fluid	Summation interval
		13° C.	NaCl 0·6, $CaCl_2$ 0·1	·0008 sec.
10	5	13·3	NaCl 0·7, $CaCl_2$ 0·01	·0018
		13·3	NaCl 0·6, $CaCl_2$ 0·1	·0010
		13·2	NaCl 0·6, $CaCl_2$ 0·1	·0005
4	10	13·6	NaCl 0·7, $CaCl_2$ 0·01	·0009
		13·6	NaCl 0·6, $CaCl_2$ 0·1	·0006

The other method was to excise the tissue and place it in a fluid rich in calcium salts, and then to observe the progressive change in the summation interval as equilibrium was reached. This was done both for muscles and for nerves. All the experiments show the summation interval becoming shorter after immersion in the fluid containing a high concentration of calcium salts. The change appears from Exps. 1, 3 and 6 to be rapid at first, and then to become slow. Exp. 7 shows that the change may reduce the summation interval to a very small value if the time allowed is very long.

Sartorius muscle of frog excited in non-neural region. Stimuli 5 % below threshold. In NaCl 0·6, $CaCl_2$ 0·1.

Exp.	Temperature	Time of immersion	Summation interval
1	12° C.	20 mins.	·0016 sec.
	13	1⅔ hrs.	·0011
	13·2	4 ,,	·0011
	12	6½ ,,	·0010
3	12·8	¼ hr.	·0019
	12·7	½ ,,	·0016
	13·5	1½ hrs.	·0014

Nerve-fibres to gastrocnemius of frog. Stimuli 5 % below threshold. In NaCl 0·6, $CaCl_2$ 0·1.

6	13·0° C.	20 mins.	·0009 sec.
	13·7	2½ hrs.	·0004
	12·6	5½ ,,	·0004
7	12·3	2½ ,,	·0005
	13·1	18 ,,	·0002

Exp. 6 shows that the summation interval for the nerve-fibres examined very soon after removal from the body may be practically as long as that for the sartorius muscle examined after it has been for a

long time in a solution containing a high percentage of calcium. Compare for example the first value given for the nerve in Exp. 6 with the value found for muscle, after 6½ hours' immersion in Exp. 1. It is for this reason that I have been careful, in comparing the summation intervals for muscle and nerve in an earlier part of this paper, to choose values obtained from the two tissues after they have been in the solution over a range of times during which the summation interval is found not to change much, namely between 1 hour and 2½ hours.

5. *The effect of temperature change.* Rise of temperature decreases the summation interval. The change is not a large one, not more, it appears, than about 30 %, for a change of temperature from 8° to 18° C. The following experimental results were obtained from the sartorius muscle excited in the non-neural region.

In Exps. 12 and 13 the muscles were excited in Ringer's fluid of the composition NaCl 0·63 %, CaCl, 0·045 %, NaCl 0·025 %, and the temperature change was effected by drawing fluid of the required temperature through the electrodes. In Exp. 11 the fluid was NaCl 0·6 %, $CaCl_2$ 0·1 %; and the temperature change was made by means of a water-bath surrounding the electrodes. The three experiments are from muscles taken from different animals.

Sartorius muscle of frog, excited in non-neural region.

Exp.	Temperature	Summation interval with stimuli 5 % below threshold	Sum. int. at t_8 / Sum. int. at t_8+10
11	8·4° C.	·0010 sec.	... 1·25
	18·2	·0008	
12	8·7	·0012	
	18·3	·0009	... 1·33
	8·8	·0012	
13	9·2	·0017	
	18·9	·0013	... 1·31
	8·5	·0017	... 1·36
	18·7	·0012	... 1·33
	9·0	·0015	

It is noteworthy that these observations show a temperature quotient which is much smaller than that which I have found from the measurement in various ways of the velocities concerned in the conducted disturbance in muscles and nerves. In such cases I have found values ranging from 1·5 to 1·9, the most usual being about 1·7.

THE PARALLEL VARIATION OF THE SUMMATION INTERVAL WITH THE CURRENT-DURATION AT WHICH THE LIMINAL CURRENT STRENGTH REACHES ITS MINIMUM.

The experiments described in the last section show that whenever we encounter a shorter limiting current-duration, whether on passing from one tissue to another under like conditions or from one condition to another in the same tissue, we find also a shorter summation interval. It is known that the limiting current-duration is shorter for the motor nerve-fibres to the gastrocnemius muscle than for the muscle-fibres of the sartorius in the same animal. This fact was shown directly for the case of the toad by my own experiments[1]. For the case of the frog it rests on the following evidence. Lapicque[2] has found the limiting duration to be shorter for the motor nerve-fibres to the gastrocnemius in the frog than in the toad; and I have shown[3] that the limiting duration is longer for the sartorius muscle-fibre of the frog than for the motor nerve-fibres to the gastrocnemius of the toad. It appears from these observations that under like conditions the durations are in the frog about 0·003 sec. for the nerve-fibre to the gastrocnemius, and 0·02 sec. for the muscle-fibre of the sartorius. We have seen above that under like conditions, and with stimuli in each case 5 % below the threshold, the motor nerve-fibres to the gastrocnemius give the summation interval of 0·0005 sec., the muscle-fibres of the sartorius 0·0015 sec.

We find a similar agreement when we compare the sartorius muscle-fibre with the ventricular muscle of the frog in the same respects. I have not previously published any experiments on the relation of current-duration to liminal current-strength in the ventricular muscle, so I give here the results of two experiments which are typical of seven made under similar conditions of temperature and bathing fluid. The Stannius heart was used, excitation being by the usual fluid electrodes containing a Ringer's fluid of the same composition as that used in my experiments on the sartorius of the frog. The method used for obtaining currents of variable duration was identical with that described in my former paper, with the exception that the pendulum[4] described there

[1] Keith Lucas. This *Journal*, XXXVI. p. 114. 1907.

[2] Lapicque. *C. R. Acad. des Sciences*, CXL. p. 801. 1905. *C. R. Soc. de Biol.* LXIV. p. 6. 1908.

[3] *Loc. cit.* and This *Journal*, XXXVII. p. 470. 1908.

[4] Cf. This *Journal*, XXXVII. p. 461, Fig. 1. 1908.

was replaced, in order that longer durations might be available, by an arm driven round on a vertical axis by means of the governed clock-work drive of a gramophone. The arm made a complete revolution in ten seconds, and the interval between the opening of the two keys was adjustable by means of a circle divided to 1000 parts, so that the duration of the current could be adjusted to the nearest 0·01 sec. The observations were always made in duplicate, first with successively increasing, then with decreasing durations, and a mean was taken. I give one set of observations in full.

Exp. 14. Ventricle of frog in Stannius standstill. 1¼ hours after placing in Ringer's fluid. Temp. = 13° C.

Duration of current Seconds	Liminal current-strength Arbitrary units
0·3	77·5
0·6	45
1·2	27·5
2·4	17
3·6	15
5·0	15
3·6	15
2·4	17
1·2	25
0·6	42·5
0·3	77·5
Mean values :	
0·3	77·5
0·6	43·7
1·2	26·2
2·4	17
3·6	15
5·0	15

This experiment shows the limiting current-duration to be about 3 sec. Exp. 15 shows the corresponding time as about 2 sec.

Exp. 15. Ventricle of frog in Stannius standstill. ¾ hour after placing in Ringer's fluid. Temp. = 12·5° C.

Mean values.

Duration of current Seconds	Liminal current-strength Arbitrary units
0·3	52·5
0·6	45
1·2	38·2
2·4	36
3·6	36

These times are very much longer than those found for the sartorius muscle-fibres of the frog. And accordingly on referring back to the

experiments on summation interval we see that under like conditions the summation interval for the sartorius muscle-fibre is 0·0015 sec., that for the ventricle 0·008.

The shortening of the limiting current-duration as the effect of calcium in the bathing fluid has been described by Mines. For example, one of his experiments[1] gives the limiting duration in the frog's sartorius as about 0·05 sec. in NaCl 0·6 and $CaCl_2$ 0·1%, while in NaCl 0·7 % it is greater than 0·12 sec. In accordance with this we find (in Exps. 4 and 10 described above) the summation interval for the sartorius muscle of the frog made practically twice as long when a solution containing only 0·01 % $CaCl_2$ is substituted for one containing the high percentage 0·1 % $CaCl_2$.

I have shown that when a frog's sartorius is excised and placed in a solution containing a high percentage of calcium the limiting current-duration becomes progressively shorter as equilibrium is reached, the change being more rapid at short times after immersion[2]. Exps. 1 and 3 show that a corresponding shortening of the summation interval goes on in the same tissue under like conditions.

Finally it may be seen from experiments made by Mines and myself that at high temperatures the limiting current-duration is shorter than at low. Compare for example the figure given for the relation of current-strength to current-duration at 17·6° C. and 9·0° C. in the sartorius muscle[3]. The current-strength has practically become steady at 0·02 sec. with the temperature 17·6° C., whereas at 9·0° C. there is still a well-marked fall going on. The same fact has been noted by Lapicque[4]. Again we find a parallel change in the summation interval. From Exps. 11, 12 and 13 given above it appears that the interval is prolonged in the ratio 1 to 1·3 by a fall of temperature from 18° C. to 8° C.

All these changes then which we know to produce an alteration in the limiting current-duration alter the summation interval in the same direction. I would lay particular stress upon the case of the high concentration of calcium salts and on the progressive changes observed after immersion in Ringer's fluid, since other time relations, such for example as the length of the refractory period, do not undergo a parallel change.

[1] Mines. This *Journal*, XXXVII. p. 439. 1908. See also an experiment by Mines in a paper of mine. This *Journal*, XXXVII. p. 472. 1908.

[2] Keith Lucas. This *Journal*, XXXVII. p. 470, Exp. 8 and Fig. 8. 1908.

[3] Keith Lucas and Mines. *Ibid.* XXXVI. p. 344, Fig. 4. 1907.

[4] Lapicque. *Journal de Physiol.* p. 631, July 1907, and *C. R. Soc. de Biol.* LXIV. p. 6. 1908.

Conclusion.

Having described the experimental facts which lead to the hypothesis that the summation interval is an expression of the same fundamental property which determines the limiting current-duration, I will examine this hypothesis in greater detail.

In an earlier part of this paper I stated that the summation interval may be regarded as a measure of the rate at which the excitatory disturbance subsides. This statement needs both explanation and justification. By excitatory disturbance I mean that local change which is directly produced by the passage of the exciting current, the change which modern theories of excitation tend to recognise as the concentration of ions at suitable surfaces within the excitable cell. The statement amounts therefore to saying that the phenomena of summation of stimuli are due to the fact that the said disturbance does not subside instantaneously when a current ceases to pass, and, if partially persisting, facilitates excitation by a second current. That something persists and makes matters easier for the second current is of course the experimental fact. The assumption which my statement contains is that the disturbance which persists and favours the second current is that identical disturbance immediately produced by the first current, the excitatory disturbance. The justification which I offer for such an assumption is that this is the simplest account which can be given of the phenomenon. There is no conducted disturbance or other recognisable change set up by an "inadequate" stimulus, so that it would be superfluous to postulate the persistence of any change other than that local change which we recognise as the immediate effect of the current. But it must be borne in mind that this assumption is made.

If we admit that a longer summation interval means a slower subsidence of the excitatory change, then on our hypothesis a tissue in which the lowest limit of the exciting current is reached at a longer current-duration is also one in which the excitatory change subsides more slowly. And the slow subsidence of the excitatory disturbance is the condition which determines the longer limiting current-duration.

This conclusion suggests at once many possible inferences with regard to the properties of different excitable tissues and the effects of various changes of conditions. In particular I am tempted to discuss the difference between those tissues which normally undergo spontaneous

excitation and those which are quiescent until roused by a definite stimulus reaching them from outside, or to attempt an analysis of the changes of excitability brought about by such conditions as temperature. But I hesitate to carry the discussion any further at present for the following reasons.

In terms of recent theories of electric excitation the excitatory disturbance which is the subject of discussion in this paper means a change of concentration of ions at suitable surfaces within the excitable cell. And the "rate of subsidence" of the excitatory disturbance will be the rate of diffusion of the ions, by which the concentration changes effected by the exciting current are opposed. It would seem therefore that we have two reasons for postponing discussion. In the first place the hypothesis put forward in this paper on experimental grounds ought to be examined in the light of that theory. And in the second place, if the two should prove conformable, then we ought to be able to replace the vague physiological expression "the rate of subsidence of the excitatory disturbance" by a physical constant; so that the comparison of different tissues and the analysis of the effects of temperature change should become quantitative instead of qualitative, a question of physics instead of physiology.

The cogency of these reasons has recently become even greater. For since the experiments described in this paper were completed, Mr A. V. Hill of Trinity College has taken up the mathematical investigation of the theory of electric excitation with a view to obtaining a modification of Nernst's theory which shall give a more complete conformity with the experimental facts than was obtained by the theory in the original form which Nernst put forward. This investigation, which will shortly be published in this *Journal*, realises the hopes expressed in the last paragraph. I shall accordingly return to this discussion in connexion with the mathematical investigation of the theory of excitation.

Summary.

In several papers published within the last four years I have shown by experiments made with exciting currents of variable duration and of variable rate of increase that there is for each excitable tissue a characteristic time-factor involved in excitation. This time-factor determines the duration at which an exciting current reaches its smallest value, and the smallest rate of increase at which a current will

excite. The time-factor is for example smaller in the motor nerve-fibres to the gastrocnemius than in the muscle fibres of the sartorius of the toad.

This characteristic time-factor has been shown by my experiments to be variable by change of such conditions as temperature and the inorganic salts contained in the fluid which bathes a tissue.

Experiments described in this paper afford a measure in various tissues and under various conditions of the maximum time interval at which two stimuli 5 % below the threshold strength will sum their effects and cause excitation. This summation interval is found to vary, as we pass from tissue to tissue and from one condition to another, always in the same direction as the time-factor mentioned above.

On these experimental facts is based the hypothesis that the time-factor of excitation and the summation interval are determined by a common function, namely the rate at which the excitatory disturbance subsides. In terms of recent theories of the excitatory disturbance this would mean the rate at which the concentration of ions brought about by the exciting current is dissipated by opposed diffusion.

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CARDIAC METABOLISM OF ALCOHOL[1].

By PHILIP HAMILL.

(From the Pharmacological Laboratory, Cambridge.)

THE disappearance of alcohol from the body, when taken by the mouth or injected into the circulation, has been the subject of numerous observations.

Bodländer[(3)] working on man and on dogs found that there was a small excretion from the lungs, skin, and kidneys. This seldom exceeded some 5 % of the total quantity administered to a man even when large in amount (50 c.c. absolute alcohol). He therefore assumed that the remainder is used by the body and burnt to water and CO_2.

Attwater and Benedict[(2)] found in the course of their metabolism experiments that, except for a small percentage, alcohol is oxidised and used isodynamically with carbohydrates; so that its energy value corresponds with the supposition that it is completely oxidised to CO_2 and H_2O.

Dixon[(1)] has shown that small doses of alcohol improve the beat of the isolated heart perfused with oxygenated Ringer's solution, especially when the perfusion has lasted for some time and the solution contains no nutriment such as sugar. He found the improvement greater in the case of a rabbit which was accustomed to receive daily injections of alcohol than in a normal rabbit, and suggests that the "immunised" animal is capable of using alcohol as a foodstuff to a greater extent than the normal animal.

The present research was undertaken to determine whether an isolated heart is capable of utilising alcohol as a foodstuff like sugar.

Estimation of Alcohol. For the purpose of these experiments a delicate method of estimation, capable of being used in very dilute

[1] This research was carried out during the tenure of the Coutts Trotter Studentship of Trinity College.

solutions, and sensitive to very small differences in the percentage of alcohol, is of primary importance. The methods which have been hitherto employed fall into two main groups, the one depending on the determination of the quantity of chromic acid reduced by a known volume of the solution of alcohol, the other on the conversion of the alcohol into ethyl iodide and the determination of the iodide formed as in the ordinary method of estimating ethoxy-groups[10]. This latter method has been used by Stritar[8] who claims to an accuracy in estimation of 0·5—1 % when working with 50 mgr. Since such large quantities are seldom available in a perfusion experiment it was necessary to fall back on the chromic acid method, which, though not ideal, can be carried out with very small quantities of alcohol.

Bodländer[5] treated the fluid with a solution of chromic acid in concentrated sulphuric acid and used the change of colour as an index of the degree of reduction.

Nicloux[6] treats the solution of alcohol with concentrated sulphuric acid, adds a solution of potassium dichromate and boils, more chromate is added and the boiling continued until the fluid retains a yellowish tinge, indicating a slight excess of chromate. He claims as accuracy of about 5 % in solutions containing 0·1 % of alcohol. His method is open to three main objections. (1) The change from blue-green to yellow-green is difficult to detect and depends on the illumination. (2) Owing largely to the escape of aldehyde the chromate used varies with the rapidity with which the mixture is heated and the time during which it is maintained at the boiling point. (3) The concentration of the dichromate solution, 19 gr. per litre, is empirical and the quantity used does not correspond to any definite stage in the oxidation of alcohol. In consequence I have found that a different value for the alcohol content of a standard solution may be obtained on different days, even when the comparison tubes recommended by Nicloux are used. So that for this method it is of the utmost importance that all comparative determinations be made at one sitting and under identical conditions.

Norris and Benedict[7] take a known volume of alcohol and add to it a considerable quantity of concentrated sulphuric acid. Aliquot parts are then heated with a known quantity of chromic acid in concentrated sulphuric acid, and the unreduced excess is determined by adding excess of ferrous sulphate and back-titrating with permanganate. Their method requires about 100 mgr. of alcohol in each sample for analysis, so that the same objection applies to it as to that of Stritar,

and further the viscosity of their solutions renders accurate measurement difficult.

The method which I have employed is most satisfactory with small quantities of solutions of alcohol of 0·05 to 0·1 %, and depends on the fact that, in the presence of a moderate concentration of sulphuric acid and slight excess of chromic acid, alcohol is oxidised quantitatively to acetic acid and no further. It is important that an excess of chromic acid be present, otherwise a considerable proportion of the alcohol escapes as aldehyde. In the oxidation of alcohol to acetic acid 46 parts of alcohol require 32 parts of oxygen corresponding to 196·3 parts of potassium dichromate. Consequently 5 c.c. of 0·1 % alcohol require 2 c.c. of a solution of dichromate of 10·78 gr. per litre.

The procedure is as follows: 5 c.c. of the solution of alcohol 0·05—0·1 % are delivered into a small flask, and an accurately measured quantity of 2—2·5 c.c. of the dichromate solution is added; 5—10 c.c. of pure sulphuric acid are now gradually added; the flask being carefully cooled the while. The mixture is then raised to its boiling point and maintained there from 2—3 minutes, or the flask may be immersed in boiling water for 5—10 minutes. The mixture is then cooled, diluted with water, and the excess of dichromate is determined. For this purpose I use a solution of ferrous sulphate with potassium ferricyanide as indicator, but in some cases I have added excess of iron and determined it by permanganate. It is convenient to have the iron solution of such strength that 5 c.c. correspond to 1 c.c. of the dichromate solution and to employ a burette graduated to 1/20 c.c.; in this way one division corresponds to 1/100 c.c. of the chromate. Since one always uses 5 c.c. of the solution of alcohol, and 2 c.c. chromate correspond to a solution of alcohol 0·1 %, it follows that n c.c. chromate correspond to alcohol $\frac{n}{2}$ parts per mille.

The duration of heating exercises little influence on the analysis provided the mixture is raised to its boiling point; and no loss of oxidising power occurs if the acid mixture is heated in the absence of alcohol, when the sulphuric acid used is quite pure. This is shown by Table I, in which the mixtures indicated were heated for different times. A solution of alcohol of about 0·8 part per mille was taken and from this a solution of about 0·64 %, was obtained by dilution.

The various titrations agree closely and the reduction found for the more dilute solution is in perfect agreement with that obtained by calculation.

TABLE I.

c.c. of $FeSO_4$ used when titrated:	2 c.c. chromate 5 c.c. H_2SO_4 5 c.c. H_2O	2 c.c. chromate 5 c.c. H_2SO_4 5 c.c. alcohol circa ·8 ‰	2 c.c. chromate 5 c.c. H_2SO_4 5 c.c. alcohol circa ·64 ‰
(*a*) Direct	9·36	—	—
	9·27	—	—
(*b*) After 1½ mins. ...	—	1·70	—
(*c*) After 2 mins.	—	1·67	3·25
	—	—	3·20
(*d*) After 3 mins.	9·36	1·68	3·17
	9·30	1·67	3·17
(*e*) After 5 mins.	—	1·60	—
	—	1·70	—
Mean	9·32	1·67	3·20
Equivalent excess of chromate ...	—	0·358	0·686
Therefore chromate used ...	—	1·642	1·314

(Calculated 1·642 × ·8 = 1·314.)

A set of analyses were next performed on solutions containing 1·0, 0·8, 0·64 per mille of alcohol, which were prepared by making up a solution of 1 % alcohol from freshly distilled absolute alcohol (sp. gr. 0·7987 at 10·5° C.) by weighing out 5 gr. and diluting to 500 c.c. This stock solution was then diluted ten times, and from this latter solutions ·8 ‰ and ·64 ‰ were prepared. The results are shown in Table II.

TABLE II.

Strength of alcohol	Chromate added	Excess of chromate found	Chromate used	Factor $\frac{\text{Chromate used}}{\text{Percentage}}$
1 ‰	2·5 c.c.	·477	2·023 c.c.	2·023
·8	2·0	·390	1·610	2·013
·64	2·0	·704	1·296	2·025
·64	1·7	·407	1·293	2·020

From these it is seen that the quantity of chromate used is strictly proportioned to the quantity of alcohol in 5 c.c. of the solution taken, and the values found differ very slightly from the theoretical value obtained from the equation:

$$\frac{\text{Number of c.c. of chromate used}}{2} = \text{Concentration of alcohol in parts per mille in 5 c.c. solution.}$$

In most of the determinations, the concentrations used have varied between 0·5 and 1 per mille, thus the quantity of alcohol taken for each

titration does not exceed 5 mgr. and variations of less than 1/20 mgr. can readily be detected.

Estimation of alcohol in tissues and perfusion fluids. A known quantity of the fluid in which the alcohol is to be determined is first subjected to distillation at ordinary pressure; if much protein matter be present it should be first precipitated by the addition of a well boiled solution of picric acid. For ordinary purposes where concentration is not required it is sufficient to connect the flask with the condenser by means of an ordinary Kjeldahl distillation trap. The proportion of alcohol which passes over as the distillation proceeds has been tested in

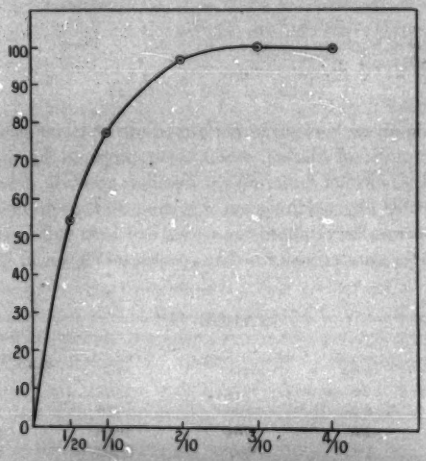

Fig. 1. Ordinate = Percentage of total alcohol in distillate.
Abscissa = Fraction of whole distilled.

a series of experiments. Quantities of 100 c.c. of ·1% alcohol were placed in the flask and submitted to distillation, a definite volume was distilled over, made up to the original volume and its alcohol content determined. The proportions of alcohol passing over are shown in the curve, Fig. 1. Before each distillation the condenser was emptied, a little distilled water was placed in the flask and rapidly boiled in order to sweep away all traces of alcohol.

In order to obtain accurate results it is necessary that the vessels used be free from all contamination with organic, and other reducing matter.

Throughout this research all the vessels used were thoroughly cleaned with hot chromic acid solution, the same pipettes (one of 5 c.c. for the alcohol, and one of 2 c.c. finely graduated for the chromate) were always used, and loss of alcohol by evaporation from the distillate was prevented by providing the condenser with a long drawn out point dipping below the surface of a small quantity of water in the receiver.

The Utilisation of Alcohol by the Heart.

The experiments were performed on the isolated heart of the rabbit perfused with oxygenated Ringer's solution at a pressure of 30—40 cms. of fluid. The animals were killed by pithing and the cannula of the apparatus was inserted into the aorta, the great veins were divided and the lungs removed. The heart and cannula were then connected with a perfusion apparatus (Locke's latest pattern) and the saline solution turned on.

Should a record of the contractions be required the ventricle may be connected, by means of a thread, with a lever writing on a smoked drum.

By this apparatus the heart and the perfusing fluid may be maintained at any desired temperature, the variations usually not exceeding 0·5° C.

In order to determine whether the heart is capable of removing alcohol from the perfusing fluid some alcohol was added to Ringer's fluid until a solution of about 0·3 % was obtained. A heart was then fixed to the perfusion apparatus in the usual manner and after it (wt. = 3·75 gr.) had been beating for an hour with ordinary Ringer's solution the alcoholic solution was substituted. The first 50 c.c. of this which came through the heart were rejected and a sample of the fluid reaching the heart was taken for analysis. It was found to contain 0·315 %. The heart was beating 120 times per minute and the rate of flow through the coronary vessels was 3 c.c. per minute. The fluid was returned to the perfusion bottle from time to time. After 1 hour, 25 minutes at 37° C. the perfusion was stopped and the fluid remaining, in all 140 c.c., was submitted to analysis. The concentration had fallen to 0·287 %, a diminution of 10 % of the total alcohol present. During the perfusion 230 c.c. had passed through the heart. A control experi-

ment was performed at the same temperature without a heart, 400 c.c. of fluid being circulated at the rate of 11 c.c. per minute. After 2½ hours the concentration had fallen from 0·40 % to 0·37 %, a diminution of 7·5 %. Thus when the heart was in the circulation more alcohol disappeared in a shorter time with a slower rate of flow.

In another experiment with alcohol in which 150 c.c. were circulated 3½ times in three hours 25 minutes analysis indicated a concentration of 0·22 % before and 0·15 % after circulation, a diminution of 32 % of the total quantity, corresponding to a disappearance of 52 mgs. of alcohol. The distillation and estimation were carried on in duplicate and the results were concordant.

In another experiment with a heart wt. 1·3 gr. in which 100 c.c. circulated five times in two hours the concentration of alcohol fell from ·915 per mille to ·525 per mille, a diminution of 44 %, corresponding to a disappearance of 44·5 mgr. of alcohol.

Since a relatively large quantity of alcohol disappears from the solution which is circulated repeatedly an experiment was made to see whether there is any appreciable diminution of the alcohol of the fluid which is collected as soon as it leaves the heart.

The heart in this experiment was in high tonus so that the individual contractions did not produce great excursions of the lever (temperature of fluid reaching the heart = 36·5° C.). Arterial and venous samples were collected simultaneously 50 c.c. of each were taken, 20 c.c. distilled water were added, and distillate was collected in 5 c.c. of distilled water and distillation was continued until the receiver (a standard 50 c.c. flask) was filled to the mark.

Arterial distillate: 5 c.c., 2·5 c.c. chromate required 4·30 c.c. Ferrous sulphate.
Venous distillate: 5 c.c., 2 c.c. chromate required 2·10 c.c. Ferous sulphate.
1 c.c. chromate = 4·90 c.c. $FeSO_4$.
∴ 4·30 c.c. $FeSO_4$ = 0·88 c.c. chromate.
2·10 ,, = 0·43 ,,
∴ Alcohol in arterial solution = 1·62 c.c. chromate, *i.e.* 0·81 per mille.
,, venous ,, = 1·57 c.c. ,, 0·785 ,,

The rate of flow through the coronary vessels was 2 c.c. per min. and the weight of the heart was 2 grms. hence the rate of disappearance of alcohol was 0·025 milligram per gram of heart substance per minute. The results of the analyses which were performed with great care have a possible error of 1 %. Of the values given that for the arterial fluid represents a minimal value and that for the venous is probably maximal. It is therefore fair to say that in this case the rate of removal of alcohol from the fluid lies between 0·02 and 0·03 milligram per minute per

gram of heart substance. Similar values have been obtained from other experiments.

If the alcohol be used as a food by the heart it will be oxidised and leave the heart in the form of oxidation products, probably as carbon dioxide and water. For complete oxidation 1 mgr. of alcohol requires 2·1 mgr. or 1·45 c.c. of oxygen, hence the consumption of 0·02 to 0·03 mgr. of alcohol would necessitate the absorption of 0·029—0·0435 c.c. of oxygen per gram per minute. In the case of the puppy's heart perfused with blood Barcroft and Dixon have shown[1] that an oxygen consumption of 0·03 c.c. per gram per minute is an average amount, but this may be greatly exceeded in increased tonus and activity. With the rabbit's heart perfused with saline solutions Brodie and Cullis[10] have found similar values so that there is sufficient oxygen absorption for the complete destruction of the alcohol.

The fact that a small quantity of alcohol added to the perfusion fluid improves the beat of a heart which has been receiving no nutriment in the perfusion fluid for some time coupled with the disappearance of the drug from the fluid is good ground for the belief that the organ is capable of using alcohol as a foodstuff within certain limits.

This consideration is supported by an experiment performed upon a rabbit which had been "immunised" to alcohol[2]. It had received daily during four days a dose equivalent to 1 gram of absolute alcohol per kilo of body weight, and for two days a dose equivalent to 1·5 grams per kilo. No dose was given later than 48 hours before the animal was killed.

The heart was perfused with oxygenated saline for three hours and at the end of this time alcoholic saline was substituted. Analysis showed the "arterial" saline to contain 0·143 % of alcohol. Venous samples collected simultaneously with the arterial showed an alcohol content 0·139%, a diminution of 0·04 mgr. per c.c. Allowing for a possible error of one unit in each of the values found the diminution must be taken as lying between 0·02 and 0·06 mgr. per c.c. An accurate value for the consumption cannot be given, since the rate of flow was not observed, but such a great diminution in alcohol content has never obtained during a single passage through the heart.

These experiments seem to justify the view that the heart at least is capable of utilising alcohol as a foodstuff. Stoklasa[8] asserts that

[1] *Journ. of Physiol.* xxxv. p. 182.

[2] I am indebted to Dr Dixon for kindly permitting me to use the heart of this animal which had been "immunised" by him.

under anærobic conditions extracts of heart pancreas, muscles, and blood are capable of breaking down glucose, and he claims to have isolated alcohol from the mixture. His extracts were obtained by pressing the organs in a Buchner press and precipitating the juices obtained with alcohol and ether. The fermentations were carried out in the presence of toluol as an antiseptic, and the organisms found in the digest were separated in pure culture and found to be incapable of causing alcoholic fermentation in anærobic conditions. Feinschmidt confirmed his results and succeeded in obtaining alcohol from mixtures in which neither ærobic nor anærobic organisms could be isolated. And although Arnheim and Rosenbaum (1) and for a time Cohnheim were unable to confirm these results, the experiments of Ransom have established the position of Stoklasa and his fellow workers.

SUMMARY.

1. A method of estimating alcohol in dilute solutions is described.
2. The isolated heart is capable of metabolising alcohol.

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CREATIN EXCRETION IN THE BIRD AND ITS SIGNIFICANCE. By D. NOËL PATON.

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Introduction. In spite of a large amount of work by many investigators the significance of creatin in the economy is still unexplained.

(1) Before 1905 creatin was generally regarded as a catabolite of muscular metabolism which is ultimately excreted in the urine as creatinin. The chief evidence upon which this view was based is that creatin is a constant constituent of muscle. But even the important investigations of Meissner and of Voit which will presently be considered afford no conclusive proofs that the production of creatin in muscle or the output of creatinin in the urine is increased during increased muscular catabolism as it should be if it is a product of such catabolism. Further, the value of most of the older observations is vitiated by the faulty and defective methods of estimation which were employed.

(2) The colorimetric method described by Folin in 1905 undoubtedly gives much more satisfactory results than any of the older methods, although possible fallacies in the results obtained have to be considered[1]. Using this method, Folin, from the examination of a large number of specimens of urines of individuals kept upon a creatin and creatinin free diet, came to the conclusion that the excretion of creatinin is a measure of the endogenous metabolism of the body[2]. Somewhat later[3] he recorded a series of experiments upon the administration of creatin by the mouth to man, which, according to him, show that the substance is not excreted as creatinin, but that it is, to a certain extent at least, retained in the body, a retention which is better marked upon a nitrogen-poor than upon a nitrogen-rich diet.

[1] See Benedict, *Influence of Inanition on Metabolism*, 1907, p. 395, and Van Hoogenhuyze and Verploegh, *Ztsch. f. physiol. Chem.* LVII. p. 165. 1908.

[2] *Amer. Journ. of Physiol.* XIII. p. 67. 1905.

[3] *Festschrift für Olaf Hammarsten*, III. 1906.

He suggests (p. 18) that creatin is to be regarded as a specialized food of some sort, but he admits that "the true state of affairs can probably not be definitely settled except by a very much longer series of feeding experiments."

Klercker[1] also states that the administration of the creatin in flesh does not increase the output of creatinin and that when large quantities are taken only from 24 to 34 per cent. reappears in the urine.

In rabbits Czernecki[2] did not find that the administration of creatin augmented the excretion of creatinin.

Lefmann[3] failed to find any conversion of creatin administered parentally into creatinin in dogs, but Van Hoogenhuyze and Verploegh in considering his results[4] show that these results do tend to demonstrate that such a conversion takes place.

Van Hoogenhuyze and Verploegh[5] found that taking 2 grams of creatin by the mouth produced a small effect on the creatin and creatinin excretion, and that the effect was in the direction of increasing the excretion of creatinin and creatin. Plimmer, Dick and Lieb[6] give a similar result.

Meissner[7] states that creatin administered hypodermically or by the mouth to dogs is entirely excreted, for the greater part as creatin and to a smaller extent as creatinin. Voit[8] also makes the statement that creatin and creatinin given to a dog by the mouth are excreted in the urine. They both used the old defective methods.

More recently Mallet[9], using a method, the limits of error of which he determined, found that when creatinin was taken in doses of from 5 to 15 grams in the day, it was all eliminated as such in the urine, and that when creatin is taken in doses of from 3 to 15 grams, it is practically all excreted as creatinin. Folin dismisses these results by saying: "Mallet's facts and mine are mutually so exclusive that I cannot undertake to give his any further consideration."

In considering these results of Folin and others, the possibility of a destruction of creatin in the alimentary canal, or its incomplete absorption, cannot be ignored. Evidence will be adduced later to show that the objection is valid (Exp. 6, p. 495).

[1] *Ztsch. f. d. ges. Biochem.* VIII. p. 59. 1906.
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[8] *Ztsch. f. Biol.* IV. p. 112. 1868.
[9] *Bull. U. S. Office of Exper. Station No.* 66. 1899.

In advancing his views as to the significance of creatin, Folin seems to me to give too little consideration to the work of older investigators and more especially does he appear to ignore the value of Meissner's work. He says, "I have carefully looked through the classical investigations of Meissner on the subject and find them surprisingly uncritical even for his own time." Meissner's papers are very remarkable, and are to my mind most suggestive. They forecast several of Folin's theories. His method of collecting the urine of the hens, upon which his observations on birds were made, was imperfect, his methods of analysis were inadequate, he rarely gives any details of his experiments and yet he arrives at conclusions which have only been confirmed and extended by the work of subsequent investigators. Thus his statement, that, in the investigation of the metabolism of creatin and creatinin, no feeding with flesh is allowable, is a clear indication that he recognised the separation of the "endogenous" and "exogenous" sources of the substances more recently emphasised by Folin.

In his work upon dogs he *assumed* that creatinin is simply a product of creatin and therefore he made no effort to separate one from the other. He states that in full feeding with flesh-free food, *i.e.* in young rapidly growing pigs, the excretion of creatin (creatinin) is at its minimum, while on insufficient diet its amount increases. This he ascribed to the rate at which muscle tissue is being used. He fully recognised and emphasised the different origin of urea and creatin (along with creatinin), a point which Folin has more recently insisted upon.

The work of Carl Voit[1], published in the same year as Meissner's investigations, appears to me to be of less value, although it too contains observations which the results of more recent investigators have substantiated. The observation that exercise does not increase the creatin of muscle as had been stated by Liebig, but that it may actually decrease it, has been recently confirmed by the investigation of Brown and Cathcart in this laboratory[2]. Like Meissner he regarded creatinin as a product of creatin. His records show that creatin may appear in the urine in fasting, an observation which has subsequently been confirmed, and that the total output is increased.

By his observation on the creatinin excretion on flesh-diet and on

[1] *Ztsch. f. Biol.* IV. p. 77. 1868.

[2] *Biochem. Journ.* IV. p. 420. 1909. Since this was in type a paper by Pekelharing and Van Hoogenhuyze has appeared (*Ztsch. f. physiol. Chem.* LXIV. p. 262), tending to show that creatin is increased in active muscle.

mixed diet he indicated, as Meissner had done, the double source—exogenous and endogenous—of the urinary creatin.

The evidence at present available yields little support to the idea that creatin is in any sense a food.

(3) The theory advanced by Mellanby[1] that the liver "is continuously forming creatinin from substances carried to it by the blood stream from other organs and that in the developing muscle this creatinin is changed to creatin and stored, while after the muscle has reached a saturation point creatinin is continuously excreted," does not rest on direct observations and may therefore be disregarded until these are forthcoming.

It is thus evident that at the present time the significance of creatin in the economy is by no means understood. The following investigations are a contribution to the solution of the problem.

Normal muscle contains creatin and not creatinin. In spite of the dubious results of many of the older investigators, the observations of Mellanby[2] seem to show that the muscle of mammals and birds contains creatin and not creatinin. Taking advantage of the solubility of creatinin in alcohol, I have tested this in the bird and in the rabbit as follows.

A duck was killed by decapitation and the left breast muscles were at once taken, and while still contracting, weighed, minced small with sharp scissors and ground in a mortar with powdered glass.

A weighed 20·2 grms.
B weighed 20·0 grms.

To each, 10 c.c. of water were added with constant rubbing and then 100 c.c. methylated spirit. The mixture was left standing for three hours then strained through calico. The residue was again extracted by rubbing in the mortar with 100 c.c. of spirit; again strained and the process repeated a third time. The alcohol extract was then filtered and made up to 325 c.c.

A was evaporated to 10 c.c. at a low pressure and at a temperature under 60° C.
B " " " " " " "

A was very opaque. To it 15 c.c. picric acid and 5 c.c. NaHO were added and after five minutes it was made up to 125 c.c. A faint brown colour which could not be read appeared. When made up to 250 c.c. no colour was visible.

B was filtered and 8 c.c. of the filtrate were treated with 5 c.c. HCl, 1 in 10, and put in the steam bath for four hours. It was then made up in the usual way with NaHO and picric acid and diluted to 500 c.c. This gave a reading of 3·5, or 0·116 per cent. of creatin as creatinin in the muscle. Alcoholic extraction is of course not calculated to remove all

[1] This *Journal*, xxxvi. p. 486. 1908.
[2] *Ibid.* xxxvi. p. 458. 1908.

the creatin. Thus this method which should have dissolved the creatinin rather than the creatin failed to extract any preformed creatinin from muscle.

The muscle of a rabbit treated in the same way failed to show any estimable amount of creatinin in 12 grms. while in 7·2 grms. the method employed, although not calculated to dissolve all the creatin, yielded ·178 per cent. as creatinin.

Amount of creatin in muscle. Dr Cathcart has been good enough to give me the following figures showing the amount of creatin (as creatinin) in the muscle of the goose[1]. His figures agree with those of Mellanby for the fowl[2].

Goose.			*Duck.*		
Leg muscle	A 0·334 p.c.		Breast muscle ...	A 0·37 p.c.	
	B 0·339			...	B 0·35
Breast muscle ...	0·32		*Goose.*		
Gizzard muscle ...	A 0·060		Breast muscle ...	A 0·32	
	B 0·061			...	B 0·316

In birds creatin takes the place of creatinin in the urine. One great difficulty in investigating the significance of creatin in mammals is the want of definite knowledge of its true relationship to creatinin. In 1908 I found that in the urine of birds creatinin is practically absent, creatin taking its place[3]. This fact seems to indicate that the problem of the significance of creatin may be investigated more simply in the bird than in the mammal, and for this reason I have studied the influence of certain factors upon its excretion.

(*a*) *Operation.* The bird was prepared by making, under full anæsthesia, an artificial anus as far from the cloacal orifice as possible. Through this the fæces escaped or were washed out by a daily enema. A dressing of cotton wool, held in position by a leather jacket which laced at the back, was kept over this orifice. Admixture of urine and fæces was thus completely prevented.

Geese, ducks and hens stand the operation well. One goose lived in the laboratory for eight months after the production of an artificial anus. Great care is required in keeping the bowel properly cleared by enemata. When these were being given it was found impossible to avoid the loss of about 20 cm. of urine per diem.

Two geese died some time after the operation of perforating ulcer of the bowel, about two inches above the artificial anus.

[1] Determined by the method described in the *Biochem. Journ.* IV. p. 421. 1909.

[2] *Loc. cit.* p. 472.

[3] A preliminary communication upon this was made to the Physiological Society in January 1909.

(*b*) *Collection of urine.* For the collection of urine the bird was kept in a special metabolism cage placed upon a wire netted floor of No. 10 standard wire (3 mm. diam.) with a mesh of 1 cm. set upon a large glazed funnel of 81 cm. diameter. The urine passed down the funnel and through a tea-strainer fastened below it to prevent feathers etc. from entering the vessel in which the urine was caught. In this strainer some of the uric acid was also caught embedded in mucus. The urine was collected, measured and filtered through glass wool. The contents of the strainer were placed in a glass cylinder and shaken violently along with the glass wool filter. The washings were then filtered through another glass wool filter—the liberated uric acid in fine suspension passing through, and these washings were combined with the original urine and the whole made up to a convenient volume.

The amount of urine passed in 24 hours could not be accurately estimated, since the birds daily spilt some of their drinking water into it. As obtained it was between 600 and 1000 c.cm., with a specific gravity of 1003 to 1006; the reaction was neutral or faintly alkaline to litmus; to phenolphthalein it was always acid.

(*c*) *Chemical methods.* For the determination of creatin and creatinin it was generally necessary to concentrate the urine. This was at first done on a steam bath in an evaporating basin, care being taken to keep the fluid just on the acid side of the neutral reaction. Later the method of evaporating *in vacuo* at 50° C. was adopted and thus the possibility of conversion of creatin to creatinin was minimised. Usually 200 c.c. were evaporated to 20 c.c. and of this 10 c.c. were used for the determination of the presence or absence of creatinin while the other 10 c.c. had 5 c.c. of 10 p.c. HCl added and were heated within the steam bath for five hours to convert the creatin to creatinin. The creatinin was determined by Folin's method with a Duboscq colorimeter. The total nitrogen was determined by Kjeldahl's method.

Repeated observation with this method upon the fresh urine and upon urine concentrated as described above demonstrated that creatin takes the place of creatinin in the urine of the bird.

I find that Meissner[1], working with the crude method of picking the fæces from the urine, dissolving the latter in alcohol and crystallizing from the solution, came to the conclusion that creatinin is not normally present in the urine of birds while creatin is present. With a method so crude and open to fallacies the conclusion is hardly justified, but the use of a more accurate method in my hands fully

[1] *Loc. cit.* p. 174.

confirms his conclusion. When, as in my earlier observations, creatinin was found to be present, this was certainly due to a conversion of creatin in the process of evaporating in an acid medium upon the steam bath instead of, as in subsequent observations, evaporating at a low pressure and at 50° C.

That the presence of creatin in place of creatinin is not the result of malnutrition, a state which in mammals is associated with its appearance in the urine, is shown by the fact that the geese fed freely on maize and plasmon and gained weight while under observation.

Average daily excretion of creatin. On the creatin-free diet given the amount of creatin excreted per day varies considerably, but as the subsequent tables show the average amount in the goose of about 4500 to 5000 grams is between 30 and 50 mg. (as creatinin)—6 to 10 mg. per kilo.

Relationship of creatin excretion to the excretion of total nitrogen. Folin[1] stated, as a result of his examination of a series of urines, that the creatinin excreted in man does not vary with the excretion of total nitrogen, when the amount of nitrogen in the diet varies. My own results on dogs and the figures more recently given by Cathcart[2] do not entirely confirm this conclusion.

On creatin-free diets in the present observations the total nitrogen metabolised varied from 0·69 to 2·14 grams per diem according to the protein richness of the diet, while the average creatin nitrogen was as follows:

Total nitrogen in grms.	Creatin nitrogen in grms.	Creatin nitrogen % of total nitrogen
·69	·0114	1·6
·85	·0123	1·4
·88	·0150	1·7
·97	·0170	1·7
1·02	·0134	1·3
1·75	·0216	0·72
2·14	·0165	0·77

These figures show that although the creatin nitrogen does not vary proportionately with the total nitrogen it tends to vary directly with it.

Is creatin an end product of avian metabolism? To obtain conclusive evidence as to whether creatin is an end product of metabolism is difficult. Certainly it cannot be answered by giving creatin by the

[1] *Amer. Journ. of Physiol.* XIII. p. 66. 1905.
[2] This *Journal*, XXXIX. p. 311. 1909.

mouth, since there is the possibility of non-absorption or decomposition in the alimentary canal. Exp. 6 shows that the administration of 0·140 gram of creatin to a goose in this way was not followed by any increased excretion in the urine.

Intravenous injection must yield fallacious results from the sudden flooding of the blood with creatin.

Subcutaneous injection should give the most reliable information, and this method of administration was selected. But even with this method it is difficult to get an accurate measure of the excretion of the injected creatin. The excretion of creatin from day to day under normal conditions varies distinctly, and hence it is not safe to assume that upon the days following the injection of the substance the normal or endogenous excretion is unchanged and that the difference between the average before and after and during the excretion gives an accurate measure of the excretion of the injected creatin. Again, the injection of creatin may produce disturbances in the metabolism. On one occasion the goose took hardly any food on the day of the injection and the difference between the actual excretion and the average amounted to considerably more than the total creatin administered. But it will afterwards be shown that a fast of even one day may raise the creatin excretion very considerably and hence in this case the amount excreted gave no measure of how much of the injected substance was eliminated.

Exp. 1. Goose A. Five months after operation weighed 4450 grms. having fallen from 5100 grms. On the 2nd day 0·063 grm. creatin was given hypodermically. On the 1st, 2nd, and 5th days the average daily excretion was 0·0508 grm. On the 3rd and 4th days 0·177 grm. was excreted, a rise of 0·0764 grm. above the average, or, if the 5th day is counted as still high from the continued excretion, the rise is to 0·0947 grm. above the average.

TABLE I.

[1] Day	Urine	Total nitrogen	Creatin	Weight	Food taken	
1	850	0·646	0·0560	4450	291	
2	1060	0·996	0·0880	—	291	0·063 grm.
3	880	0·609	0·0962	—	309	creatin
4	900	0·678	0·0808	—	295	
5	1500	1·260	0·0600	4200	335	

Exp. 2. Duck operated on one month previously weighed 1800 grms.

On the 3rd day creatin equal to 0·063 grm. creatinin determined by Dubosc's colorimeter was injected hypodermically. The excretion of urine fell on the two succeeding days and the animal took only about a half of its food on the 2nd day after the injection.

[1] In all the tables the day indicates that on which the urine was collected at 10 a.m. This is the urine of the previous 24 hours. Food taken is that taken in previous 24 hours.

TABLE II.

Day	Urine	Total nitrogen	Creatin	Weight	Food	
1	700	0·490	0·0333	1800	170 grms.	
2	900	0·567	0·0363	1700	170	
3	1000	0·742	0·0202	1750	170	0·063 grm. creatin as creatinin
4	650	0·582	0·0623	1680	170	
5	450	0·382	0·0487	1770	95	
6	850	0·559	0·0484	1800	170	
7	1100	0·816	0·0321	—	170	
8	400	0·291	0·0145	—	170	
9	600	0·574	0·0231	1800	170	

The average excretion on the 1st, 2nd, 3rd, 7th, 8th and 9th days was 0·0271 grm. per diem. On the 4th, 5th and 6th days 0·1594 grm. was excreted, 0·088 grm. above the normal.

Exp. 3. Goose operated upon on Oct. 27th. Put up Oct. 30th. Weight 5000 grms. Food, maize and water. At 4 p.m. on the 4th day, about 0·0400 grm.* of creatin reckoned as creatinin in 5 c.cm. water was injected subcutaneously.

TABLE III.

Day	Urine in c.cm.	Total nitrogen in grm.	Creatin	
1	1050	0·735	0·0393	
2	950	0·545	0·0437	
3	900	0·617	0·0492	
4	1000	0·686	0·0497	0·04 grm. creatin injected
5	950	0·572	0·0537	
6	1100	0·631	0·0734	
7	1000	0·602	0·0480	
8	1000	0·602	0·0510	
9	900	0·630	0·0495	

Average excretion of creatin as creatinin on 1, 2, 3, 4, 7, 8, and 9 days =	0·0472
Excreted on 5th and 6th days	0·1271
Average excretion for these two days	0·0944
Excess over normal	0·0327
Injected	0·0400

* A small loss from the syringe occurred during the injection.

These experiments give no indication that in the bird under normal conditions creatin undergoes a change in the body. They show that it is to be regarded as an end product of metabolism.

Influence of fasting on creatin excretion. As a first step to the solution of the question of the significance of creatin it seemed desirable to study the effect of withdrawal of food, since in this condition the muscle substance, the great creatin holding tissue of the body, is drawn upon to a greater or less extent according to the previous

nutrition of the animal. Further it has been now definitely proved that in mammals fasting is accompanied by the appearance of creatin along with the creatinin in the urine. This may be due to creatin or a precursor being a substance available for reconstruction in the muscle when a suitable supply of oxygen and carbohydrates is available, and that when these are not forthcoming it is thrown out; or it may be due to the decreased activity of the enzyme, described by Gottlieb and Stangassinger[1], by which creatin is normally converted into creatinin. The behaviour of creatin in birds in this condition is therefore extremely interesting.

Exp. 4. Goose B, a young growing bird, had an artificial anus made on Nov. 7th. It was put in the cage on the 20th and fed on maize meal 300 grms., water 150 c.c. with 15 grms. of plasmon. Of this it took daily about three-quarters. It weighed 4300 grms. It fasted on the 2nd, 3rd, and 4th days inclusive. On the 5th day 30 grms. of plasmon were added to the food.

TABLE IV.

Day	Urine in c.cm.	Total nitrogen	Creatin	Creatin Total	Nitrogen % total N.	Weight	Food
1	1000	1·57	0·0558	0·0308*	1·32	4300	Maize + 15 grms. plasmon
2	2370	1·44	0·0427	0·0159	1·11	4200	0
3	1530	0·994	0·0491	0·0182	1·83	3750	0
4	940	0·771	0·0357	0·0132	1·71	3650	0
5	1280	2·07	0·0238	0·0089	0·43	4250	Maize + 30 grms. plasmon
6	1560	2·08	0·0141	0·0053	0·26	4250	,, ,, ,,

* First day in cage. In most experiments it was found that the excretion of creatin on this day was high.

Exp. 5. Goose A, in which an artificial anus had been made on Nov. 7th when it weighed 4250 grms., was put in the cage on Jan. 12th, when it weighed 5100 grms. It was fed upon maize 230 grms. with 170 c.c. of water added. On the 5th, 6th, and 7th days it fasted.

TABLE V.

Day	Urine in c.cm.	Total nitrogen	Creatin	Creatin Total	Nitrogen % total N.	Weight	Food
1	1000	0·994	—	—	—	5100	Maize
2	900	0·844	—	—	—	—	Maize
3	700	0·740	0·0237	0·0088	1·19	—	Maize
4	900	0·843	0·0306	0·0114	1·35	—	Maize
5	850	0·752	0·0351	0·0132	1·76	—	0
6	1000	0·714	0·0676	0·0252	3·53	—	0
7	1200	0·789	0·0980	0·0366	4·64	4750	0
8	1500	1·070	0·0440	0·0164	1·53	—	Maize

[1] *Ztsch. f. physiol. Chem.* LII. p. 1. 1907.

Exp. 6. Goose A had been in the laboratory with an artificial anus from Nov. 7th to May 24th. It had fallen in weight from 5800 grms. in Jan. to 4200 grms. The food was maize 200 grms. with 150 c.c. water, to which casein was added upon the days indicated. On the 16th, 17th, and 18th days the bird fasted. On the 3rd day it had 140 mgs. of creatin in 50 c.cm. water by the stomach tube.

TABLE VI.

Day	Urine in c.cm.	Total nitrogen	Creatin	Creatin Nitrogen Total	% T. N.	Weight	Food taken		
1	1000	0·635	0·0326	0·0121	1·90	4200	0		
2	1360	1·14	0·0356	0·0134	1·18	4200	330		
3	1970	1·98	0·0414	0·0155	0·78	4250	365		140 mgs. creatin in 50 c.cm.
4	1220	1·22	0·0360	0·0135	1·10	4200	369		water by stomach tube
5	1270	1·47	0·0296	0·0110	0·75	—	365	50 grms. casein with food	
6	1270	1·26	0·0261	0·0098	0·77	4150	368		
7	1450	1·52	0·0210	0·0078	0·50	4250	355		
8	1160	1·35	0·0334	0·0125	0·92	4300	355		Faces in urine
9	1080	1·30	0·0703	0·0262	2·01	4300	365		Urine had peculiar smell *
10	1060	1·10	0·0580	0·0216	1·96	4300	270		
11	1500	1·39	0·0450	0·0168	1·21	4380	305	Maize alone	
12	1080	0·92	0·0292	0·0109	1·19	4300	315		
13	1200	0·936	0·0380	0·0142	1·52	4350	305		
14	1260	1·04	0·0276	0·0102	0·99	4300	265		
15	1260	0·995	0·0312	0·0116	1·16	4300	307		
16	1560	1·102	0·0906	0·0338	3·06	4150	0		
17	1560	1·419	0·2340	0·0873	6·15	4050	0		
18	1000	1·11	0·1440	0·0537	4·84	3900	0		
19	1500	1·62	0·0759	0·0283	1·75	4300	365	50 grms. casein in food	
20	1180	1·19	0·0894	0·0147	1·24	4150	353		

* There seems to have been a toxic condition possibly due to casein in food.

Exp. 7. The bird weighing 2500 grms. had an artificial anus made upon Sept. 29th. It was put in the cage on Oct. 1st. On the 2nd, 3rd, and 4th days it fasted.

TABLE VII.

Day	Urine in c.cm.	Total nitrogen	Creatin	Creatin Nitrogen Total	% total N.	Weight	Food taken in grms. about
1	980	0·621	0·0659	0·0246	3·76	2250	100
2	1000	0·518	0·0557	0·0208	4·00	2200	0
3	800	0·448	0·0773	0·0288	6·43	2150	0
4	900	0·491	0·0828	0·0309	6·30	2100	0
5	1450	0·802	0·0780	0·0291	3·6	2150	160
6	1300	0·491	0·0516	0·0192	3·9	2050	very little food taken
7	1200	0·638	0·0879	0·0328	5·1	—	little food taken
8	1450	0·589	0·0536	0·0200	3·4	1980	most of food taken

Expts. 8 and 9 show similar results (p. 502).

Thus during fasting there is generally an increase in the excretion of creatin, relatively to the total excretion of nitrogen, and under certain conditions an increase which is absolute. This increase is most manifest when the animal is not well nourished. These results indicate that creatin is a catabolite of muscle tissue. In experiments 4, 8 and 9 a very low output of creatin is to be observed upon the days immediately following the fast.

Meissner[1] records a similar increase in the excretion of creatin and states that after 36 hours without food a hen passes more creatin in the later hours than in the earlier. In dogs he states that there is a rise in the creatin + creatinin excretion, an observation which corresponds with Dorner's results in the rabbit, but which has not been obtained in the study of prolonged fasts in man.

In discussing the significance of the creatin excretion in fasting the following facts must be kept in mind. The skeletal muscles of the goose contain about 0·33 p.c. of creatin (as creatinin), *i.e* 0·134 p.c. of creatin nitrogen. The total nitrogen in muscle is about 3·6 p.c. The creatin nitrogen is thus 3·7 p.c. of the total nitrogen. The other tissues of the body, so far as they have been investigated, contain only traces of creatin.

The increase in the amount of creatin must indicate either (1st) an increased catabolism of muscle by which more creatin is set free, or (2nd) some interference with a process by which creatin is disposed of in the body, either (*a*) by resynthesis into muscle substance or (*b*) by decreased activity of an enzyme *creatinase* by which it is normally split up, as is suggested by Gottlieb and Stangassinger, or (3rd) a qualitative change in the metabolism of muscle by which more creatin is formed from a given quantity of muscle by the diverting of a portion of the nitrogen which would otherwise not be excreted as creatin.

Were it the case that in fasting there is simply a failure of a normal resynthesis of creatin (see Fig. 1, B) or of some precursor of creatin, then the catabolism of "flesh" calculated from the creatin nitrogen excreted (Fig. 1, A) might possibly equal, but could not exceed, the catabolism as calculated from the total nitrogen (Fig. 1, TN), unless, concomitantly with the decrease in the resynthesis of creatin or its precursor, an increase in the synthesis of non-creatin nitrogen took place, a condition which appears highly improbable. Such an excess occurs in many cases, and hence it seems justifiable to exclude a decreased resynthesis as the cause of the rise.

[1] *Loc. cit. supra.*

If in fasting there were simply a deviation of so much nitrogen to the increased formation of creatin in muscle (Fig. 1, E), then some proportion between the increase in the production of creatin and the decrease in excretion of non-creatin nitrogen should be manifest. An analysis of Exps. 5 and 6, where the increase in creatin is most marked, fails to demonstrate any such relationship and shows a direct rather than an inverse relationship between the output of creatin and total nitrogen.

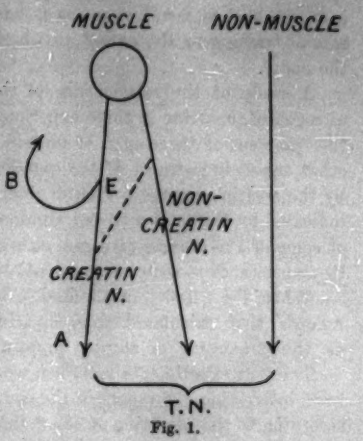

Fig. 1.

Exp. 5.

Non-creatin N.	Creatin N.
·740	0·0088
·843	0·0114
·752	0·0132
·714	0·0252
·789	0·0366

Exp. 6.

Non-creatin N.	Creatin N.
1·068	·0338
1·333	·0873
1·057	·0537

The experimental results recorded on pp. 491–2, showing that creatin is an end product of avian metabolism, negative the possibility that the increased excretion in fasting is due to a failure in the action of a hypothetical creatin splitting enzyme.

The exclusion of these hypotheses leaves only the view that the increased creatin excretion indicates an increase of muscle catabolism, and, since creatin is an end product, from the amount excreted conclusions may be drawn as to the amount of muscle "flesh" catabolised.

The previous study of the changes in the body during fasting have shown that the essential tissues live upon the non-essential and that muscle wastes very markedly. In the prolonged fast of the salmon the transference of the nitrogenous constituents of the muscle to the growing ovaries and testes has been definitely demonstrated. That muscles may waste and part at least of their substance be retained in the body

is an established fact, but so far it has not been possible to trace the rate of wasting or the extent to which their substance is retained in the body[1].

A study of the relationship of the creatin nitrogen to the total nitrogen of the urine in these experiments throws important light upon this problem of the course of protein metabolism in the muscles and other organs in fasting. If the muscle "flesh" catabolised as indicated by the creatin excreted is greater than the total "flesh" disintegrated as indicated by the total nitrogen eliminated, this must mean a retention of some of the muscle nitrogen, either by resynthesis in the muscle or by transference to some more essential organ.

When the "flesh" catabolised, calculated from the total nitrogen, exceeds that calculated from creatin nitrogen the stored nitrogen, *i.e.* the circulating or surplus protein of Voit, is being catabolised.

The four experiments recorded, when examined in detail, bring out some interesting differences in the course of metabolism during fasting according to the condition of the animal. (See Table VIII.)

In Exp. 4, in a fat young growing gander fed on plenty of maize and plasmon before the fast, the amount of muscle waste as indicated by the creatin nitrogen excreted remained low throughout the three days' fast. The excretion of total nitrogen indicated a much greater protein catabolism which in this case may well have come from proteins of non-muscular source, such as the circulating protein of Voit.

In Exp. 5, in a gander which was no longer growing and which had been fed on a comparatively nitrogen-poor diet, on the first day of the fast the "flesh" catabolised calculated from the total nitrogen was much larger than the muscle catabolised as calculated from the creatin. Non-muscle protein was being used. On the second day the amounts were about equal. Muscle is the source of the nitrogen. But on the third day the amount calculated from the creatin nitrogen exceeded that calculated from the total. Hence muscle nitrogen was being retained in the body.

In Exp. 6, in a gander which was considerably reduced, while upon the first day the catabolism of flesh calculated from total nitrogen was higher than that calculated from creatin nitrogen—non-muscle protein was being used—on the second and third day the difference was the other way, and hence muscle nitrogen is being retained.

In Exp. 7, in a duck upon a low diet, this last condition is reached

[1] See This *Journal*, xxxiii. p. 1. 1905.

upon the first day of the fast, and becomes more marked as the fast progresses. A retention of muscle nitrogen occurs throughout.

These results may be tabulated as follows:

TABLE VIII.

Flesh calculated from Total Nitrogen and Creatin Nitrogen.

Day		From total nitrogen	From creatin nitrogen	Course of metabolism
Exp. 4.				
1		43	15	Food protein used.
2		40	11·7	Non-muscle protein used.
3	Fast	27	13	„ „ „ „
4		21	9·9	„ „ „ „
5		57	6·4	Food protein „
6		56	3·8	„ „ „
Exp. 5.				
4		23	8·3	Food protein used.
5		29	9·7	Non-muscle protein used.
6	Fast	19	18	Balance.
7		22	27	Retention of muscle nitrogen (·5 grm. flesh).
8		29	10	Food protein used.
Exp. 6.				
15		27·6	8·7	Food protein used.
16		30·6	25	Non-muscle protein used.
17	Fast	41	61	Retention of muscle nitrogen (20 grms. flesh).
18		30	38	„ „ „ (8 grms. flesh).
19		45	20·6	Food protein used.
20		33	10·7	„ „ „
Exp. 7.				
1		17	18·3	Balance.
2		14	20	Retention of muscle nitrogen (6 grms. flesh).
3	Fast	12	21	„ „ „ (9 grms. flesh).
4		13·6	23	„ „ „ (9·4 grms. flesh).
5		22·2	21·7	Balance.

The method of studying the course of metabolism in fasting thus arrived at in the bird may be applied to the investigation of the same process in the mammal if creatinin is regarded as a derivative of the creatin of muscle and, along with creatin excreted, taken as a measure of muscle catabolism.

Meissner and Voit undoubtedly believed that it is to be so regarded and that the amount excreted is a measure of muscular catabolism. Van Hoogenhuyze and Verploegh came to the same conclusion although they seem to believe that creatin may undergo

a change into some other substances, basing their conclusion upon the autolytic and perfusion results of Gottlieb and Stangassinger. The results of these observers appear to me to be unsatisfactory and inconclusive. They fully admit the conversion of creatin into creatinin, but the failure to recover the total amount of creatin and creatinin has forced them to postulate the presence of enzymes breaking down these two substances. The question requires investigation by other methods.

The autolytic experiments of Van Hoogenhuyze and Verploegh[1] seem to show that creatinin is an end product, while all the results of administering creatinin[2] clearly indicate that it is excreted unchanged.

For Folin's theory of the independence of creatin and creatinin I can see very little evidence, and, if creatinin is a product of the dehydration of creatin, it must come from the endogenous metabolism of *muscle* and not from the general protein metabolism of the body.

The fact that throughout the early days of a fast the creatinin + creatin nitrogen excreted corresponds very closely to 3·7 p.c. of the total nitrogen, the proportion which creatin nitrogen bears to the total nitrogen of muscle is an additional consideration in favour of the origin of creatinin from muscle.

Benedict[3] suggests that the preformed creatin eliminated in the urine during fasting is an index of the flesh (muscle) catabolised. But this view seems to leave out of account the fact that, when muscle is catabolised apart from fasting, no creatin appears in the urine, and further it fails to appreciate the significance of the fact that in fasting, in man at least, as the creatin rises in amount, the preformed creatinin falls—a very strong indication that the one is a precursor of the other and that in fasting there is merely a failure in the complete conversion of creatin to creatinin.

To summarize—the evidence that creatin is changed to creatinin, the fact that in the early days of a fast the creatinin nitrogen excreted bears to the total nitrogen the same proportion which it does in the muscle, and the evidence that creatinin is an end product all indicate that the excretion of creatinin, or in fasting of creatinin + creatin, is a measure of the muscular catabolism in the mammal.

The evidence we at present possess seems to indicate that the

[1] *Ztsch. f. physiol. Chem.* LVII. p. 209. 1908.

[2] See Van Hoogenhuyze and Verploegh, *Ibid.* LVII. p. 161. 1908; Folin, *Festsch. f. Hammarsten*, p. 9. 1906.

[3] *Loc. cit.* p. 459.

capacity of the liver and other tissues for converting creatin to creatinin is strictly limited and that it may readily be overstepped.

The influence of variations in the activity of the dehydrating enzyme need not here be considered as in two of the fasts recorded the calculations are made from the creatinin + creatin results, and even in the case of La Tosca the failure to reckon the creatin introduces a possible error only in the later days of the fast.

In the following table the flesh catabolised in the fasts recorded by Cathcart on Beauté[1], by Van Hoogenhuyze and Verploegh on La Tosca[2] and by Benedict[3] has been calculated in this way.

TABLE IX.

Day of Fast	Flesh from total nitrogen	Muscle from creatin nitrogen	
Beauté (Cathcart, *loc. cit.* p. 121).			
Last day of Egg and Milk	450	390	Non-muscle flesh used.
3rd day	380	320	Muscle flesh chiefly used.
6th day	320	300	„ „ „
14th day	210	320	Retention of muscle nitrogen (110 grms. flesh).
Starch & cream 3rd day	77	320	Marked retention of muscle nitrogen (140 grms. nitrogen).
Eggs &c. 2nd day	260	280	Balance.
La Tosca (Van Hoogenhuyze and Verploegh, *loc. cit.* p. 469).			
Day of Fast	—	—	
3rd day	290	420	Retention of muscle nitrogen (110 grms. flesh).
6th day	210	430	„ „ „ (220 grms. flesh).
14th day	110	320	„ „ „ (210 grms. flesh).
S. A. B. Benedict (*loc. cit.* p. 392).			
Day of Fast	—	—	
1st day	340	340	Balance.
4th day	320	360	Slight retention of muscle nitrogen (40 grms. flesh).
7th day	280	350	Retention of muscle nitrogen (70 grms. flesh).

They show a very steady catabolism of muscle throughout the fasts, as indicated by the excretion of creatinin nitrogen, while a steady decrease in the "flesh" catabolised, as indicated by the excretion of total nitrogen, shows the retention or resynthesis of the non-creatin yielding part of the muscle.

Dorner's[4] observations on the influence of fasting in rabbits show

[1] *Biochem. Ztsch.* VI. p. 109. 1907.
[2] *Ztsch. f. physiol. Chem.* XLVI. p. 415. 1905.
[3] *The Influence of Inanition on Metabolism*, 1907, p. 392.
[4] *Ztsch. f. physiol. Chem.* LII. p. 225. 1907.

results corresponding more closely with mine in birds than with those obtained in man. The progress of the fast is characterised by an enormous rise in the output of creatinin + creatin nitrogen, a rise due entirely to the increased output of creatin. With the increased protein waste of fasting the output of total nitrogen is also increased. In his third experiment, in which water was not withheld, the average excretion of nitrogen for seven days before the fast was 1·25 gram and of creatinin + creatin nitrogen 0·0346 gram, while from the fourth to the tenth day of the fast the nitrogen output averaged 2·18 grams and the creatinin + creatin nitrogen 0·106 gram. Calculating the "flesh" metabolised from these figures the following results are arrived at:

	Non-muscle flesh	Muscle flesh	
Before Fast	35	26	Food protein used.
During Fast	60	79	Muscle nitrogen retained.

The influence of carbohydrates on the creatin excretion in the bird. An interesting question is that of the effect of carbohydrates upon the excretion of creatin, since Cathcart has adduced very cogent evidence

Exp. 8. Goose A, weighing 4550 grms., put in cage on 10th Feb. It fasted on the 1st, 2nd and 3rd days. On the 3rd day it had 20 grms. of glucose in 100 c.c. of water.

Day	Urine	Total nitrogen	Creatin	Creatin Nitrogen Total	Creatin Nitrogen % of total N.	Food	
1	1000	0·78	0·072	0·0269	3·4	0	
2	900	0·78	0·063	0·0235	3·0	0	
3	1110	0·699	0·044	0·0163	2·3	Glucose	
4	1000	0·980	0·026	0·0097	0·99	Maize and water	
5	800	0·966	0·013	0·0048	0·50	,,	,,
6	1150	—	0·0287	0·0107	—	,,	,,

Exp. 9. Goose B, put up 10th May. Weight 4000 grms. It fasted on the 3rd, 4th, and 5th days. On the 4th day it had 20 grms. of glucose in 100 c.c. water by stomach tube. Food, maize 230 grms, with 150 c.c. water.

Day	Urine	Total nitrogen	Creatin	Creatin Nitrogen Total	% T.N.	Weight	Food taken	
1	1370	0·858	0·0464	0·0174	2·03	4000	311	
2	1910	0·858	0·0464	0·0174	2·03	4100	330	
3	1060	0·530	0·0457	0·0170	3·21	4050	0	
4	980	0·471	0·0490	0·0182	3·86	4000	0	20 grms. glucose.
5	1580	0·695	0·0600	0·0224	3·22	3990	0	
6	850	0·731	0·0323	0·0120	1·64	4000	350	15 grms. plasmon with maize.
7	1000	0·860	0·0294	0·0084	0·98	4050	205	,, ,, ,,
8	1290	0·903	0·0214	0·0080	0·89	4200	335	,, ,, ,,
9	1090	0·981	0·0255	0·0095	0·97	4200	320	,, ,, ,,
10	1260	1·04	0·0170	0·0063	0·61	4300	337	,, ,, ,,
11	1370	1·26	0·0178	0·0066	0·52	4200	296	,, ,, ,,

that as protein-sparers these occupy a pre-eminent position, a theory which receives strong support from the figures shown in the preceding table of the relationship of the excretion of total nitrogen and creatin nitrogen during feeding with starch and cream at the end of the fast; see Table IX where the retention of nitrogen was equivalent to no less than 312 grams of flesh. The following experiments on this subject were performed.

TABLE X.

Flesh calculated from total nitrogen and creatin nitrogen in grms.

	From total nitrogen	From creatin nitrogen	
	Exp. 8.		
1 Day of Fast	20	19	
2 ,, ,,	20	17	
3 ,, ,,	19	12	glucose.
Maize	27	7·1	
,,	26	3·5	
	Exp. 9.		
Maize & Plasmon	26·6	12·6	
1 Day of Fast	14·6	12·4	
2 ,, ,,	13·0	13·3	glucose.
3 ,, ,,	19·0	16·3	
Maize & Plasmon	20·3	8·8	
,, ,,	24·0	6·2	

These two experiments show the same increase in the excretion of creatin in fasting as is shown in Exps. 4 to 7, and they further show that under the conditions of these experiments, the influence of the administration of glucose is not so manifest as might have been anticipated. In the first experiment a trace of glucose appeared in the urine showing that the limit of tolerance had been overstepped, and in the second experiment about 20 c.cm. of the whole was vomited by the goose.

Taking the relationship of creatin nitrogen to total nitrogen and the "flesh" catabolism indicated by each, the influence of the administration of 20 grams of glucose seems to be in the direction of limiting muscular and non-muscular catabolism alike.

A more satisfactory answer might be expected from a study of the influence of the substitution of an isodynamic supply of proteins and of fats for carbohydrates. Did the sugar exercise a simple sparing action, then the isodynamic amount of protein or fat should have the same effect.

Conclusions.

In the urine of birds (goose, duck and hen) creatin takes the place of the creatinin of mammalian urine.

Creatin is an end product of the catabolism of avian muscle and the amount excreted thus gives a measure of the muscle catabolised.

In fasting the excretion of creatin is generally increased both absolutely and also proportionately to the excretion of total nitrogen.

The excretion of creatin varies directly but not proportionately with the excretion of total nitrogen.

The relationship between the excretion of nitrogen in creatin and of the total nitrogen affords information as to the course of metabolism in the body during fasting. If the "flesh" catabolised as calculated from the total nitrogen is greater than the "flesh" calculated from the creatin nitrogen, non-muscle protein is being utilized. But if the flesh calculated from the creatin nitrogen is greater than that calculated from the total nitrogen, then some of the muscle nitrogen must be retained in the body either by resynthesis in the muscles or in other organs.

In well nourished birds and during the first day of the fast non-muscle "flesh" is catabolised. In poorly nourished birds and later in the fast muscle flesh is chiefly catabolised and some of the nitrogen is retained possibly to be resynthesised.

Taking the creatinin and creatin excretion in the mammal during fasting as an index of muscular catabolism and comparing it with the total "flesh" catabolism as indicated by the total nitrogen excretion, it is found that during the course of a fast at first the non-muscle "flesh" is used and that later there is a retention of muscle nitrogen in the body.

The administration of glucose in fasting seems to have no specific action on the excretion of creatin nitrogen.

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PROCEEDINGS

OF THE

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June 26, 1909.

The effects of previous forced breathing and oxygen inhalation on the distress caused by muscular work. By C. G. DOUGLAS and J. S. HALDANE.

In a paper on the Regulation of Breathing (This *Journal*, XXXVIII. p. 430) we have discussed the causes of the respiratory distress following severe muscular exertion. We wish to record here the results of some further experiments on the effects of forced breathing, and of inhalation of oxygen, before the exertion. These effects were tested by making a definite and severe muscular exertion lasting a definite time, either without previous preparation or just after moderately forced breathing for 3 minutes or quiet inhalation of oxygen for 3 minutes. During the minute or more immediately following the exertion the breathing was either recorded quantitatively, or measured by means of a large gas meter, or the whole of the expired air was collected in a large bag, measured and analysed. The pulse was also counted, and the subjective feelings of distress observed.

The conclusions arrived at may be briefly stated as follows:

1. The forced breathing has the effect of greatly diminishing the respiratory distress, the amount of air breathed during the minute succeeding the exertion, and the amount of CO_2 given off.

2. The respiratory quotient is much lower than it is if there has been no forced breathing.

3. The pulse-rate is less rapid after the exertion, and returns more quickly to normal.

4. The previous breathing of oxygen has only a very slight, if any, effect on respiration or pulse, or distress, unless the breathing has been more or less forced.

We attribute the diminished respiratory and cardiac distress to the fact that a considerable amount of CO_2 has been washed out of the body by the forced breathing. In consequence of this fact the muscular exertion does not give rise to such a large excess of CO_2 in the arterial blood as would otherwise be the case; and consequently neither the respiratory centre nor the heart is so strongly excited.

The absence of any appreciable effect from the previous normal breathing of oxygen is exactly what might be expected from the fact that breathing oxygen has no effect on normal respiration, and that the available evidence, which has recently been again discussed by Zuntz (*Die Kraftleistungen des Thierkörpers*, Festrede, Berlin, 1908), is definitely against the theory that oxygen is stored in the body when pure oxygen is breathed. Hill's recent observations as to the effects of previous inhalation of oxygen in diminishing the distress caused by muscular exertion can, we think, be explained as a result of the deep breathing which is apt to occur in persons to whom oxygen is administered. This deep breathing seemed to us very evident in the case of a boat's crew to whom oxygen was recently administered by Dr Hill at Oxford.

The following experiments may be quoted:—

Volume of air (measured by meter) breathed in first two minutes immediately following muscular exertion (running up and down 40 ft. of stairs twice in 70 seconds).

	After exertion		Total for 2 mins. cub. ft.
	First min. cub. ft.	Second min. cub. ft.	
C. G. D. resting breathing = 0·6 cub. ft. per 2 mins.	—	—	—
No preparation before exertion	—	—	3·475
Moderate forced breathing for 3 mins. ...	1·41	0·89	2·30
No preparation	2·01	1·145	3·155
Quiet breathing of O_2 for 3 mins.	1·82	1·105	2·925
No preparation	1·70	0·92	2·62
Moderate forced breathing for 3 mins. ...	1·27	0·87	2·14
J. S. H. resting breathing = 0·685 cub. ft. in 2 mins.	—	—	—
No preparation before exertion	2·11	1·54	3·65
Moderate forced breathing for 3 mins. ...	1·52	1·00	2·52
No preparation	2·12	1·56	3·68
Quiet breathing of O_2 for 3 mins.	2·22	1·57	3·79

Pulse-rate immediately after muscular exertion (running up and down 40 ft. of stairs twice). Each tenth beat was recorded by a signal on a revolving drum with time-marker. Successive times in seconds during which 30 heart beats occurred.

C. G. D.					J. S. H.			
Exercise preceded by 3 mins. moderate forced breathing. Some voluntary breathing during exertion	No preparation	Exercise preceded by 3 mins. moderate forced breathing. No voluntary breathing during exertion	No preparation	Exercise preceded by 3 mins. quiet breathing of O_2	Exercise preceded by 3 mins. moderate forced breathing	No preparation	Exercise preceded by 3 mins. moderate forced breathing	No preparation
17	13	15	11	12	14	14	15	13
18	14	18	11	13	16	14	16	13
21	17	23	15	17	17	15	15	13
31	19	26	17	17	18	16	16	14
34	19	30	18	19	19	18	18	14
31	20	33	17	20	19	18	16	14
	19		19	20	19	20	17	16
	22		20	21	20	19	18	16
	21		20	21	21	20	18	15
	23		19	21	21	20	18	16
	24		20	22			19	17
	24		21	22				17
	23		22	25				17
			20	27				
At rest before this, 30 heart beats in 27″ 26″			22					
			At rest before this, 30 heart beats in 27″	At rest before this, 30 heart beats in 26″				
Successive experiments, June 8.		Successive experiments, June 9.			Successive experiments, June 8.		Successive experiments, June 9	

As voluntary breathing on the stairs during the exertion appeared to have a marked influence on the subsequent pulse-rate, the following additional observations as regards this point were made on C. G. D. The number of beats in each half-minute after the exertion are recorded.

	First 30″	Second 30″	Third 30″	Fourth 30″
Forced breathing 3 mins. and voluntary breathing during the exertion	—	42	33	31
Forced breathing 3 mins. and no voluntary breathing during the exertion	—	54	42	36
No preparation	—	54	48	45

Previous preparation before exertion	Expired air collected in bag during first minute after muscular exertion lasting 45". C. G. D. Volume cub. ft.	CO_2 %	O_2 %	Respiratory quotient	Total amounts of gaseous exchange during first min. in cub. ft. CO_2	O_2
Nil	1·93	3·55	17·33	0·98	0·068	0·069
3 mins. forced breathing[1]	1·10	4·18	15·88	0·80	0·046	0·057
3 mins. forced breathing[2]	1·265	4·28	15·72	0·80	0·054	0·067
Nil	1·755	3·81	17·04	0·97	0·066	0·068
3 mins. quiet breathing of O_2	1·755	3·73	17·13	0·98	0·064	0·066

It will be noted that the expired air contained a larger percentage of CO_2 in the forced breathing experiments. The hyperpnoea was thus more efficient, though much less in amount, after the forced breathing.

	Alveolar air just after exertion lasting 45", preceded by moderate forced breathing for 3'. C. G. D. CO_2 %	O_2 %	Respiratory quotient
Some voluntary breathing during exertion	5·28	13·43	·67
No voluntary breathing during exertion, and actual breathing seemed very slight ...	6·43	10·89	·58
Normal alveolar air, C. G. D.	5·57	(14·75)	—

It will be noted that there was no very excessive fall in the oxygen percentage.

Quantitative graphic records were also taken of the breathing, but are not reproduced here. They were obtained with the help of the large "coffin" plethysmograph described by Haldane and Priestley. The subject, however, breathed into and out of the plethysmograph, instead of being enclosed in it. These records showed that after the forced breathing the frequency of the respirations was about a third less than when there had been no forced breathing, whether or not the subject had breathed oxygen previously. For instance, in one experiment the frequency during the first half-minute was reduced from 40 to 27 per minute, the depth being about the same.

Miss Florence Buchanan kindly took four electro-cardiograms of C. G. D., the records beginning 30 seconds after running twice up and down 30 feet of stairs, this being preceded in two instances by moderate forced breathing for 3 minutes. The results were as follows.

Successive periods of time in seconds during which 10 heart-beats occurred:

Exercise preceded by forced breathing	No preparation	Exercise preceded by forced breathing	No preparation
4·78	4·11	4·64	3·96
5·04	4·40	4·91	4·24
5·35	4·67	5·08	4·58
6·09	5·10	5·71	4·76
			5·36

[1] Breathing kept up to some extent intentionally during the exertion. Owing to this fact the alveolar air probably contained more O_2 and less CO_2 when breathing into the bag was started, and the calculated amounts of O_2 absorbed and CO_2 given off are thus lower than in the third experiment.

[2] Breathing left to itself during the exertion and therefore shallow.

A new method for the estimation of lactic acid in urine. By J. H. RYFFEL.

The method consists of three operations, but the whole procedure can be completed within two hours.

The urine is first steam distilled with rather over 50 % sulphuric acid, when the lactic acid yields acetaldehyde quantitatively according to the equation:

$$CH_3.CHOH.COOH = CH_3.CHO + H.COOH.$$

The acid distillate is then rendered just alkaline and redistilled. To the distillate Schiff's reagent (rosaniline hydrochloride bleached with sulphur dioxide) is added and the depth of colour compared by means of a Duboscq colorimeter with that obtained with the reagent and standard formaldehyde solution. The actual procedure is as follows:

1st Stage. The delivery tube of a 500 c.c. Jena distillation flask is so bent, that it can be attached to a good vertical condenser, when the flask is sloped at an angle of 45°. 40 c.c. of the urine is placed in this flask and 45 c.c. of nitrogen free sulphuric acid added rapidly from a dropping funnel, whilst the flask is shaken and cooled under the tap. The flask is then fitted with a rubber cork carrying a thermometer and an inlet tube for steam, so arranged, that, when the flask is in position, both dip well into the liquid. The flask is then placed on wire gauze and attached to the condenser, whilst the inlet tube is attached to an ordinary steam generator. The receiver of the condenser is a flask of about 300 c.c. capacity, immersed in ice or cold water, and fitted to the condenser with a loose cork, or pushed right up against the base of the condenser jacket. A gentle stream of steam is then led into the flask, which is vigorously heated with a bunsen. When distillation has begun, which should be at a temperature rather below 140° C., the current of steam is reduced to a minimum and the flask rapidly heated to about 155° C. The current of steam is then increased, and the heat applied to the flask is adjusted to maintain this temperature with a variation of about 2° either way. When about 100 c.c. has collected, or when the distillation has lasted nearly 30 minutes, the decomposition is complete.

2nd Stage. The contents of the receiver are rendered just alkaline with 2 % caustic soda, using litmus indicator, and made up with water to about 150 c.c. If on standing for a couple of minutes the liquid becomes acid again it should be again made alkaline, and so on. The condenser is washed out, and a flask with a 100 c.c. mark in the neck is placed as receiver with the same precautions as before. The neutralised distillate is redistilled till about 50 c.c. has collected. This second distillate is then stoppered and placed in a bath at about 15° C. for a short time.

3rd Stage. A standard solution of formaldehyde is prepared as follows: 10 c.c. of commercial 40 % formaldehyde is diluted to 100 c.c. This solution will keep practically indefinitely, and should be standardised by the Iodine method of Romijn. For use this solution is diluted 100 times, when it should contain nearly ·4 mg. formaldehyde in 1 c.c. and will keep practically unaltered for a week. The Schiff's reagent is prepared by adding 100 c.c. of water to 1 gm. rosaniline hydrochloride (finely powdered) and passing in sulphur dioxide from a syphon, till the dye just dissolves to a yellow solution, when the liquid is very nearly saturated with sulphur dioxide.

The day previous to the determination a series of stoppered 100 c.c. flasks is prepared containing ·5 c.c. of the reagent and 2 c.c., 3 c.c., 4 c.c., 5 c.c. respectively of the dilute standard formaldehyde solution, made up to 100 c.c. with water. These are placed in a dark cupboard till required. The colour develops very slowly, but will keep unaltered for 2 days after the first 12 hours.

To the distillate from Stage 2 is added ·5 c.c. of the reagent, the volume is made up to 100 c.c. with water, and the contents of the flask well mixed.

The flask is then stoppered and placed in a glass vessel containing water at 15° C. and left in diffuse daylight for 30 minutes. With acetaldehyde the colour comes fairly rapidly, but proceeds to fade slowly as soon as the maximum is reached.

For the colorimetric determination the same depth of the aldehyde liquid is compared with the two formaldehyde liquids, which are nearest to it in concentration of colour.

The calculation is best described by an example.

	Formaldehyde 3 c.c.	x	Formaldehyde 4 c.c.
Readings of equal depth of colour	2·42 cm.	2 cm.	1·46 cm.
10 ÷ readings	4·13	5	6·85

These figures represent the relative concentrations of colour of the solutions employed.

Then x is equivalent to $3 \text{ c.c.} + \frac{5 - 4{\cdot}13}{6{\cdot}85 - 4{\cdot}13} = 3{\cdot}32$ c.c. of formaldehyde solution per 100 c.c.

40 c.c. of the urine therefore contain lactic acid equivalent to $3{\cdot}32 \times a$ milligrammes of formaldehyde where a is the standard value of 1 c.c. of the dilute formaldehyde solution, and should be nearly ·4 mg.

If on mixing with the reagent the distillate gives a colour much greater than that in the 5 c.c. formaldehyde flask, it may be rapidly diluted to say 200 c.c. and a proportionate amount of the reagent added, or another distillation with less urine and water added to make up 40 c.c. must be undertaken.

The reagent loses sulphur dioxide rather readily, so that it must be kept closely stoppered, and must be resaturated occasionally. The standard flask with ·5 c.c. of the reagent and 5 c.c. of the dilute formaldehyde should be of such a depth of colour, that by the colorimeter 1·5 cm. with a variation of ·2 cm. either way is equivalent to ·7 cm. $\frac{N}{100}$ potassium permanganate (the colour is not a close match). If the reading is less than this, more sulphur dioxide must be passed in, if greater, sulphur dioxide must be allowed to escape.

The method was standardised with recrystallised calcium lactate with the result that

·4 mg. formaldehyde = 3·435 mg. lactic acid.

Assuming this result other observations gave the following:

Lactic acid used	Found
12·6 mg.	11·93 mg.
25·6	24·46
12·80	12·40
36·98	36·57

The accuracy of the conversion of lactic acid into acetaldehyde was tested by making solutions of 'absolute aldehyde' and comparing by the colorimetric method above with formaldehyde, but a certain amount of loss of the acetaldehyde was inevitable in measuring out the solution, etc.

The result of two series was that ·4 mg. formaldehyde was equivalent to 1·765 mg. acetaldehyde, which would come from 3·61 mg. lactic acid.

The figure for lactic acid direct is 3·435 mg.

The difference represents manipulative loss in dealing with the standard acetaldehyde solution.

Observations on normal urine point to the presence of a small quantity of lactic acid, about 6 mg. per 100 c.c. (sp. gr. 1020). The amount present varies in accordance with the specific gravity of the urine.

Finally, known lactic acid added to urine can be almost entirely recovered. For instance:

5 c.c. of a lactic acid solution gave on determination 10·92 mg. lactic acid.

When added to 35 c.c. urine the result was 12·27 mg. lactic acid.

The urine contained 2·196 mg. in 35 c.c.

Therefore lactic acid recovered from urine = 10·074 mg.

The Regulation of expiratory force during phonation. By W. A. AIKIN.

The important question, how to control the breath when using the voice has, I believe, not yet been fully discussed in the Physiological Laboratory.

Assuming that a full breath is taken in by the combined action of raising the ribs and drawing down the diaphragm in due proportion, then the expiratory forces available for phonation are:

1. Elasticity of lungs—on relaxing the diaphragm.
2. Abdominal muscular force—pressing the diaphragm upwards.
3. Elasticity of lungs and chest wall—on relaxing the rib muscles.
4. Depression of ribs.

When all these forces act together, there will be a rapid emptying of the lungs, beginning with a maximum of expiratory force, as in ordinary

quiet breathing in which elasticity plays the principal part. But if, as in phonation, the period may have to be prolonged for 15—20 seconds, it becomes necessary to keep the breath forces under control.

The gradual and even exercise of all the forces together may be possible, but it is easier to control them by using them separately.

It is obviously undesirable to depress the ribs while the diaphragm remains contracted, for that would cause excessive distension of the abdomen.

On the other hand, the relaxation of the diaphragm (1) and the abdominal muscular force (2), can be safely used before the ribs are allowed to come down. In well developed breathing the lower ribs relax (3) only towards the end of the period, and the actual depression of the ribs (4) need not take place at all.

Therefore I would suggest the distribution of the available forces in the above order as an advantageous manner of controlling the output of the breath in phonation.

By retaining the costal air in reserve the air-pressure is made more even and continuous, according to the common principle of a "dome," and also the rigidity of the expanded ribs supports the vocal pitch, and favours resonation in the neck.

A simple apparatus for demonstrating and practising the regulation of breath force is here shown. It consists of a glass tube immersed by counterpoise to any required depth in a column of water. The tube is provided with a T piece through which the air may be allowed to escape, thus imitating the passage of air through the glottis—or which may be closed and the air driven out through the bottom of the tube.

The water column is not more than 20 inches (500 mm.), for I have not been inclined to venture upon such high pressures as are indicated by McKendrick's observations (Schäfer's *Physiology*). Although a pressure of 20 inches, and more, is easily reached, it is evident that it cannot be sustained for any length of time without some interference with the circulation. The form of breathing described above is, moreover, especially well adapted to the resistance of pressure—for the weakest spot, the diaphragm, is supported by abdominal contraction—and the chest walls are made rigid by the contraction of the intercostals.

Observations on cases of cyanosis due to congenital heart disease. By H. S. FRENCH, M. S. PEMBREY and J. H. RYFFEL.

Cases of cyanosis due to congenital disease of the heart offered opportunities for the study of the regulation of respiration by the gaseous composition of the blood. In such patients there is evidence of a chronic deficiency of oxygen and compensation for this defect is shown in the great increase in the number of the red corpuscles per cubic mm. of blood, in the increased percentage of hæmoglobin and the large pulmonary ventilation.

Five cases of typical morbus cæruleus have been investigated. The composition of the alveolar air, determined by Haldane's method, shows a low percentage of carbon dioxide, especially in those patients who exhibited the most marked signs of defective compensation. The following table gives the chief data; the first three cases were young men, the remaining two young women.

No. of Case	Date	Alveolar air			Red corpuscles per cubic mm.	Percentage of hæmoglobin	Volume in litres of air breathed per minute	Number of respirations per minute	Pulse-rate per minute	Blood-pressure in mm. Hg.
		% CO_2	% O_2	$\frac{CO_2}{O_2}$						
I.	6. viii. 04	4·6	14·9	·76	13,200,000	145	—	—	—	—
		4·8	14·4	·78						
	4. xii. 05	4·29	14·48	·66	8,810,000	174	11	22	96	—
II.	20. xi. 05	4·21	14·92	·70	10,900,000	163	7½	26	—	95
		3·65	16·42	·80						
	18. ii. 08	3·04	17·36	·84	11,000,000	150				
		3·03	17·18	·80						
III.	8. v. 06	3·86	16·44	·85	11,790,000	138	7½	12	—	95
		3·72	16·68	·87						
IV.	4. v. 07	2·68	17·31	·73	12,930,000	130	6½	18	100	92
		2·54	17·46	·73						
V.	19. i. 09	3·34	16·77	·80	6,650,000	138	—	19	80	88
		3·32	15·78	·74						
		3·10	16·92	·77						
		3·19	16·91	·79						

The effects of breathing oxygen by means of a mask and valves were observed upon the colour, respiration and alveolar air; the results varied and further investigation is needed to explain the differences. The blue colour of the extremities appeared to be chiefly due to a slow

passage of the blood through the capillaries and veins. The patients did not suffer from breathlessness during rest but exertion brought it on at once in every case except the first.

The urine of two of the cases was examined for lactic acid.

No. of case	Specific gravity of urine	Lactic acid per 100 c.c.
II.	1012 (day)	6·86 mg.
	1013 (night)	3·74
III.	1021 (day)	24·6
	1021 (night)	14·85

Both of the patients were up and doing only light work during the day, but, as they were not in the Hospital at the time, it was impossible to obtain the total urine for the day and night.

Observations upon the urine of a healthy man gave the following results:

Specific gravity of urine	Lactic acid per 100 c.c.
1021 (night)	7·68, 6·27, 6·316 mg.
1019 (day)	5·68
1014·5 ,,	4·724
1013 ,,	3·97

The lactic acid in mg. per hour for the day of 15½ hours = 4·34, 3·55.
,, ,, ,, ,, night of 8½ ,, = 1·614, 1·89, 2·36.

The urine of two patients in the wards and kept in bed all day was examined:

Case of Pleuritic Effusion	Urine of day	1017	sp. gr.	6·13 mg.	lactic acid per 100 c.c.
	Urine of night	1017	,,	5·41 ,,	,, ,,
Case of Angina pectoris	Urine of 24 hrs.	1013	,,	24·11 ,,	,, ,,
				19·7 ,,	,, per hour.
2 days later when the patient was quite comfortable.	Urine of 12 hrs.	1016	,,	7·02 ,,	,, per 100 c.c.
	day			3·51 ,,	,, per hour.

The relations of secretin and enterokinase to the ferments of the pancreas. By J. MELLANBY and V. J. WOOLLEY.

(*Preliminary communication.*)

The experimental results recorded in this note deal with the amylolytic and milk clotting ferments of the pancreas. The amylolytic power was estimated from the time taken by a definite quantity of the solution to convert 3 c.c. of ·33 % starch into achroodextrin. The rennin power was determined from the time taken by a definite quantity of the solution to coagulate 3 c.c. of milk to which calcium chloride had been added to the extent of ·5%. On the assumption that proteolytic ferments coagulate milk provided they are supplied with suitable conditions, the determination of the rennin power of the solutions gave a measure of their trypsin content.

An antiseptic autolysis of an ox pancreas in water at 38° C. yielded a solution which contained a large quantity of amylopsin and rennin (·1 c.c. of the solution converted 3 c.c. of starch into achroodextrin in two minutes; and the same quantity of solution coagulated 3 c.c. of milk in one minute). An ox pancreas autolysed in glycerine (75 %) yielded an extract which contained a quantity of amylopsin similar to that contained in the water extract but only a trace of milk clotting ferment (·1 c.c. of the glycerine extract converted 3 c.c. of starch into achroodextrin in one minute; but the same quantity of solution required two and a half hours to coagulate 3 c.c. of milk). The addition of enterokinase to this glycerine extract resulted in the production of an intense rennin activity. ·25 c.c. of an enterokinase solution and ·75 c.c. of water were added to 1 c.c. of the glycerine extract. This solution was incubated at 30° C. and after various intervals of time the power of ·2 c.c. of the mixture to coagulate 3 c.c. of milk was determined. The following results were obtained:

	Coag. time for 3 c.c. milk	Tested after
·1 c.c. Original Extract	2½ hours	—
·2 c.c. Extract + Enterokinase	21 minutes	1 hour
	2 ,,	5 ,,
	¾ ,,	20 ,,

Dilution of the glycerine extract of the pancreas with water resulted in a production of rennin activity, but at a much slower rate than that

caused by the enterokinase. The following results illustrate this point. 1 c.c. of the glycerine extract was diluted with varying quantities of water to volumes of 2 c.c., 3 c.c., 4 c.c., 5 c.c., and 6 c.c. After 24 hours the rennin activities of quantities of these solutions corresponding to ·1 c.c. of the original solution were determined. The milk coagulation times in minutes were 102, 75, 63, 45, and 16—the shortest coagulation time being produced by the most dilute solution.

It appeared possible that the pancreas contained enterokinase which was not dissolved out by the glycerine and that the insolubility of the enterokinase in this solvent accounted for the water extract of the gland containing an active milk clotting ferment, whilst the glycerine extract contained only the precursor of this ferment. But this possibility was negatived by the fact that a glycerine extract of the mucous membrane of the intestine contained a large quantity of enterokinase which quickly generated rennin when added to a glycerine extract of the pancreas.

From these results it appears probable that the conversion of the rennin zymogen into an active milk clotting ferment by means of enterokinase involves an hydrolysis, and that this hydrolysis can be slowly effected by water only. The fact that an antiseptic autolysis of the pancreas yielded an extract containing large quantities of all the ferments indicates that secretin does not combine with precursors of various types contained in the pancreatic cells to form active ferments or their zymogens.

PROCEEDINGS

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October 23, 1909.

Acceleration of the mammalian heart-beat by stimulation of the vagus nerve. By H. H. Dale, P. P. Laidlaw, and C. T. Symons.

(*Preliminary Communication.*)

It has been stated by several observers (Schiff, Rutherford, Arloing) that the vagus contains accelerator fibres for the heart, which can be detected if the inhibitors are eliminated by degenerative section or by atropine.

A few years ago we observed marked acceleration of the cat's heart-beat on stimulating the peripheral end of the divided vagus after injection of appropriate doses of tropine: subsequently a similar effect was obtained in a few experiments with nicotine. The investigation was temporarily abandoned on account of the uncertainty of the results. Recently we have determined more accurately the conditions, particularly as regards the anaesthetic and the dose of the drug used to depress the inhibitor action, which enable the effect to be produced in the cat with certainty.

The effect is somewhat complex. When the peripheral end of the cut vagus of the cat is faradised, after intravenous injection of 50—100 mgms. of tropine, or 1—2 mgms. of nicotine, or certain other drugs, the effect on the heart, during the application of the stimulus, is either *nil* or slight acceleration. On the cessation of the stimulus the beat

gradually becomes slower, apparently in response to a delayed inhibition. A renewal of the stimulus during this period of slowing causes immediate acceleration, succeeded by delayed inhibition in an accentuated form when the second stimulus is stopped. The phenomena of acceleration during the stimuli and inhibition in the intervals can then be reproduced indefinitely, both phases being facilitated by repetition. After a few stimuli the acceleration during stimulus usually carries the rate of the heart-beat beyond that which existed before the first stimulus: there is apparently, therefore, a true acceleration, and not merely interference with a persistent inhibition.

The nature of the phenomenon is still under investigation, but certain obvious possibilities may be excluded.

(1) The effect is not due to spread of current on to the sympathetic accelerators. Acceleration is obtained equally well whether the stimulus is applied just below the ganglion of the trunk or low in the neck. In one experiment a stimulus so weak that it failed to produce acceleration, during a period of delayed inhibition, when applied to the annulus of Vieussens, immediately accelerated the heart when the electrodes were transferred to the vagus of the same side. Pinching the vagus nerve below the point of stimulation completely abolishes the effect.

(2) The effect is not due to sympathetic fibres descending in the vagus from the superior cervical ganglion or recurrent from the inferior cervical or stellate ganglion, since extirpation of all these ganglia and subsequent degeneration does not affect the phenomenon.

We have succeeded in producing a similar effect in the dog in a very weak form, in the ferret moderately well, in the rabbit not at all.

Reversed activity of tissue extract manufactured at high temperatures. By B. J. Collingwood.

J. Mellanby states (*Journal of Physiology*, Vol. xxxviii.) that a watery solution of thrombokinase is heat stable. Such a statement can by the nature of things be true relatively only; in this instance a temperature of 100° C. was the criterion he adopted. I find, however, that when the influence of higher temperatures is investigated some noteworthy results ensue. A tissue extract made in an autoclave at a temperature of 115° C. not only has no coagulant power but also

possesses marked *anti*coagulant activity. In the experiments detailed below watery tissue extracts of the thymus gland of the sheep were employed.

(*A*) Watery tissue extract free from residue unheated + equal bulk of freshly drawn blood, incubated at 37° C.

Coagulation in 30 *seconds.*

(*B*) Watery tissue extract free from residue heated for 1 hour at 115° C. + equal bulk of freshly drawn blood, incubated at 40° C.

Coagulation in $1\frac{1}{4}$ *minutes.*

(*C*) Freshly drawn blood manipulated in similar manner (sham mixing) but nothing added + equal bulk of freshly drawn blood, incubated at 40° C.

Coagulation in $1\frac{1}{4}$ *minutes.*

(*D*) Watery tissue extract with undissolved residue heated for 15 minutes at 115° C. + equal bulk of freshly drawn blood, incubated at 37° C.

Coagulation in 10 *minutes.*

(*E*) Watery tissue extract with undissolved residue heated for 30 minutes at 115° C. + equal bulk of freshly drawn blood, incubated at 37° C.

? Clot in 20 *minutes.*

(*F*) Watery tissue extract with undissolved residue heated for 1 hour at 115° C. + equal bulk of freshly drawn blood, incubated at 37° C.

No coagulation in 30 *minutes.*

Conclusions.

1. A temperature of 115° C. destroys the coagulant in watery tissue extract. (*A* and *B*.)

2. A temperature of 115° C. does not produce an anticoagulant in watery tissue extract free from residue. (*B* and *C*.)

3. A temperature of 115° C. does produce an anticoagulant in a mixture of watery tissue extract with undissolved residue, and the amount of this anticoagulant is directly proportional to the length of exposure to this temperature. (*D*, *E* and *F*.)

4. The anticoagulant is produced by the action of the high temperature on the undissolved residue. (*B* and *D*, *E* and *F*.)

The tissues composing a nerve trunk. By F. O'B. ELLISON.

A quantitative estimation of the tissues present in the peripheral nerves was made in the following way.

Transverse sections about 10μ thick are prepared and outline drawings made of the bundles of fibres, medullary sheaths and axis cylinders, using magnifying powers of $\times 25$ and $\times 1000$—1500. The sketches were made with Abbe's drawing camera. Some of the sections were also photographed.

The areas covered by each tissue were cut out, dried and weighed. These weights correspond to the sectional area of each tissue, and assuming the nerves to be uniform in diameter for considerable distances and all the tissues composing it to be of the same specific gravity, the weight of each tissue present will be proportional to the sectional area. The following figures were obtained in this way from medullated (median) and non-medullated (splenic) nerves of the horse.

Any non-medullated fibres present in the median were counted as axis-cylinders.

Splenic

Connective tissue	61 %
Axis cylinder	39 %
	100

Median

Connective tissue	66 %
Medullary sheath	25 %
Axis cylinder	9 %
	100

To find out whether any large error was present in the method the following control was adopted. Portion of a fresh nerve was taken and weighed. It was then carefully cleaned from adherent connective tissue (epineurium) and weighed again. The difference was equal to the amount of connective tissue removed. Parts of the same nerve, before and after cleaning, were fixed and sectioned, drawings being made as above described, and the percentage of connective tissue removed by cleaning estimated, as above, from the weight of the area of paper

occupied by it in the sketch. Appended are comparative figures showing the result.

	By direct weight	By weight of area in drawing
Nerve not cleaned	3·710	0·552
Nerve cleaned	3·025	0·429
Connective tissue removed ...	0·685	0·123
	= 18·4 %	= 22·3 %
	of total nerve not cleaned.	

Further Note on the Chemical Nature of Albinism. By GEO. P. MUDGE.

IN the Proceedings of this Society for March 1909, I gave an account of the effect upon the pelage of placing albino or piebald rats into a solution of 10 % formalin in 75 % spirit. The pelage was turned into a brilliant yellow, the reaction occurring in a short interval. At the same time I failed to obtain any reaction with mice. The following experiments show that a colour reaction may, however, be obtained from mice, by a slight modification in the experimental procedure.

EXP. I. A fawn-coloured piebald mouse placed into weak nitric acid in water, after several days manifested no change. It was then washed and placed into formalin (about 10 %?). After several weeks it was examined and all white parts had turned to a brilliant yellow inclining to orange. The claws were more intensely coloured and were orange. It was then placed into 5 % nitric acid in 75 % spirit and five days later the yellow was still present though just perceptibly fainter. This experiment was merely of a tentative nature.

EXP. II. Two fawn-coloured and two black-coloured piebald mice, male and female in each case, were placed in 5 % nitric acid in 75 % spirit, in four separate vessels and placed in the dark. Five days later they were examined. Result: the white areas of the following surfaces of the body had turned to a beautiful rose-pink colour:

Dorsal surface, chest and throat, perceptibly fainter than elsewhere.

Belly and sides more intensely; the sides rather more intense than the belly.

Head and snout much fainter.

The tarsi, carpi, claws, hairs on tail and the whiskers apparently not stained at all.

In the case of the fawn-coloured piebald pair, the male was not quite so intensely coloured as the female. But in the black-piebald pair such difference was not observable.

In all four mice the skin itself remained perfectly white.

Microscopical examination of the hairs singly, revealed no perceptible colour.

Exp. III. Treated an albino mouse (pink eyes) as in experiment 2. The result was the same; the whole pelage—with the exceptions named in exp. 2—turned a rose-pink colour, and the forehead and snout were perceptibly fainter than elsewhere; this of course may be due to the fact, that the hairs in this region are shorter than in other parts of the body. This explanation does not, however, apply to the dorsal surface of the piebald forms. It is possible that in them, the hairs though longer are slightly less numerous on the dorsal than on the lateral or belly surfaces.

Exp. IV. A female black-piebald mouse was placed in equal volumes of 10 % formalin and 75 % spirit. Five days later there was no change. It was then placed into 5 % nitric acid in 75 % spirit. Four days later there was no change. On the ninth day, all the parts described in experiment No. 2, had changed to a rose-pink colour, but not quite so intense as in that series.

Exp. V. Placed one of the mice from exp. No. 2, into equal volumes of 10 % formalin and 75 % spirit, after well washing in water. The others were retained in the original solutions. In the former case the pink colour begins to fade almost immediately after immersion, and by four days afterwards, it has completely disappeared and does not reappear. In the latter mice the colour is still retained, now three months since the experiment commenced. The mice have been kept in the dark.

Neither of the two solutions used produced any visible change in egg-albumin, in the cold, except to coagulate it.

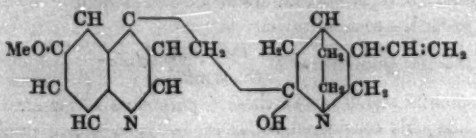
Action of Cinchona Alkaloids on Muscle. By V. H. VELEY and A. D. WALLER.

IN previous communications[1] to the Society the method of testing the relative toxicity of various poisons by means of their comparative action upon the direct excitability of the sartorius muscle of the frog has been described and demonstrated. A full description of this method and of the general procedure followed, as well as of the precautions to be observed, has been given in connection with an account of the relative anæsthetic powers of alcohol, chloroform and ether[2]. Its applicability to measurements of the physiological effects of alkaloids and glucosides has been shown in the cases of Muscarine (*Proceedings*, Nov. 21, 1908), Digitaline (Dec. 19, 1908) and Aconitine (in the press) and of many other alkaloids of which the records are not yet collated.

In our present communication we give the results of experiments upon muscle with the five alkaloids of the *Cinchona* group, viz. *Quinine*, *Quinidine*, *Cinchonine*, *Cinchonidine* and *Cinchonamine*.

Of these the first four, obtained from Cinchona bark, are known to be derived from conjoint piperidine and quinoline residues.

Quinine and quinidine are represented as follows:



Cinchonine and cinchonidine differ from quinine and quinidine in containing a hydrogen atom in place of the methoxyl (OMe) group.

Cinchonamine differs from cinchonine and cinchonidine in containing two hydrogen atoms more. It is obtained from a distinct genus, *Remijia purdiana*.

Physico-chemical data go to show (1) that *quinine* and *quinidine* are stronger bases than *cinchonine* and *cinchonidine*, and that of the first named pair, *quinine* is rather stronger than *quinidine*, (2) that as regards the two residues, *piperidine* and *quinoline*, *piperidine* is the stronger,

[1] *Proc. Physiol. Soc.*, June 20, July 18, Oct. 17, Nov. 21 and Dec. 19, 1908.

[2] *Proceedings of the Royal Society*, June 1909.

and appears to be modified (reduced in action) in all four cases by its conjunction with *quinoline*, the weaker residue[1].

Cinchonamine, partly by reason of its sparing solubility and that of its salts, partly by reason of its rarity, has not been examined by physico-chemical methods.

Our physiological data are altogether in conformity with (1); as regards (2) our experiments are not yet completed. As far as we have seen so far, the toxicity of Piperidine and Quinoline has not been sensibly different from that of Quinine.

Concentration in saline	Quinine	Quinidine
$\frac{N}{100}$ 0·324 %	Abolition and contracture in 2 minutes, no recovery.	Abolition and contracture in 4 to 5 minutes, no recovery.
$\frac{N}{400}$ 0·081 %	5 minutes, no recovery.	12 minutes, partial recovery.
$\frac{N}{1000}$ 0·032 %	6 minutes, fair recovery.	—

∴ *Quinine* is about twice as toxic as Quinidine in correspondence with the fact that its affinity value is the higher.

Cinchonine and *Cinchonidine* with a considerably lower affinity value than *Quinidine* in N/200 (0·294 %) abolish contractility in 14 minutes, *i.e.* have approximately equal effects with each other and with that of *Quinidine* at N/400, *i.e.* are about half as toxic.

Thus so far the scale of toxicity is

$$Quinine = 100 \quad Quinidine = 50 \quad \left.\begin{matrix} Cinchonine \\ Cinchonidine \end{matrix}\right\} = 25.$$

Cinchonamine stands out in these experiments as far stronger than the four alkaloids considered, so far as has been shown previously by See and Bochefontaine who gave the relation between Cinchonamine and Quinine as 6 to 1[2].

at N/1000 (0·030 %) abolition in 14 and 11·5 mins.
at N/2000 (0·015 %) „ 13·5 and 13·5 mins.

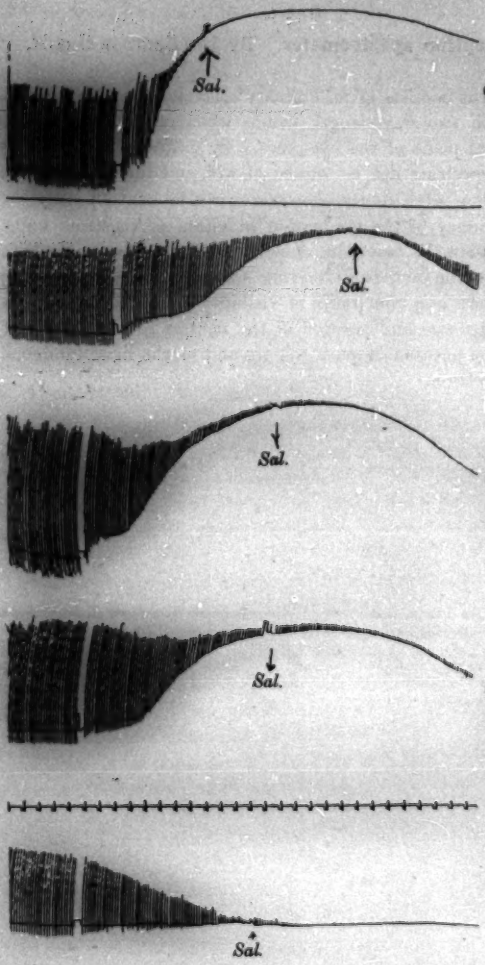
We conclude from comparison of times of abolition and recovery that the relation of strength by molecules of these two substances is $\frac{1}{4}$.

The scale of our five substances comes out therefore

Cinchonamine 400, Quinine 100, Quinidine 50, Cinchonine 25, Cinchonidine 25.

[1] Berthelot and Gandechon. *Comptes Rendus*, CXXXVI. p. 128. 1903. Veley. *Journ. Chem. Soc.* XCVIII. p. 214. 1908. XCIV. p. 758. 1904.

[2] *Comptes Rendus*, C. pp. 866, 664. 1885.


Sal.
Quinine hydrochloride
N/400 (0·081 % as base)
Sal.
Quinidine hydrochloride
N/400 (0·081 % as base)
Sal.
Cinchonine hydrochloride
N/100 (0·294 % as base)
Sal.
Cinchonidine hydrochloride
N/100 (0·294 % as base)
Sal.
Cinchonamine hydrochloride
N/2000 (0·015 % as base)

Colour-perception spectrometer. By Dr Edridge-Green.

This instrument consists of an ordinary spectrometer with a single prism, fitted with two wave-length drums which work two shutters placed in the focal plane of the eye-piece. By means of the shutters any part of the spectrum can be viewed at will, and the wave-lengths of the edges of the patch under observation can be read off from the drums. The accuracy of the graduation is within 5 Ångstrom units. With this instrument the exact size of portions of the spectrum which appear monochromatic (and their varying size with different persons) can be ascertained; also the limits of visibility on both sides of the spectrum, the exact size and position of the neutral band in different dichromics and the position of pure colours and of the most luminous portion of the spectrum.

PROCEEDINGS

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December 11, 1909.

On the Formation of Bubbles in the Vessels of Animals submitted to a Partial Vacuum. By LEONARD HILL, F.R.S. and M. GREENWOOD, junior.

Many years ago, Hoppe-Seyler asserted that if animals were rapidly exposed to a partial vacuum gas bubbles were formed in their vessels just as happens on sudden decompression from pressures greater than one atmosphere. Paul Bert, who performed a large number of such experiments, denied that bubbles were ever formed under these conditions.

We have carried out similar experiments, placing the animals under a bell-jar connected with a Geryck Pump and a manometer. In one experiment we obtained Hoppe-Seyler's result.

A rabbit was decompressed in 3 minutes to 50 mm. Hg., at which pressure it died. The body swelled enormously during rarefaction. *Post Mortem*, the heart was found to be full of air and all the large vessels contained numerous air bubbles.

On the other hand, no bubbles were seen in 8 mice, 2 guinea-pigs, a cat and a kitten treated in the same way.

The conclusion seems to be that, in general, Bert's contention is just but that, in large animals, bubbles may occasionally be produced during rarefaction.

It will be remembered that Robert Boyle saw a bubble within the eye of a viper exposed to a partial vacuum by means of his air pump.

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Hoppe-Seyler. *Müller's Arch. f. Anat. Phys. u. Wissenschaft. Med.* p. 63. 1857.
Bert. *La Pression Barométrique*, p. 937.

On the presence of a reducing endo-enzyme ("reductase") in liver and kidney. By D. FRASER HARRIS.

(*Preliminary communication*)

Press-juice (through a Klein's press) of liver (sheep) and of kidney (ox) contains a reductase which will reduce soluble Prussian blue to the pale green or colourless chromogen (blue restored at once by H_2O_2), sodium sulphindigotate and methylene blue to the pale green condition.

Juice is kept aseptic under toluol with which it is frequently shaken.

This is the endo-enzyme responsible for "the reducing-power of living animal tissues" which I studied in 1897 and in greater detail in 1906 (Biochemical Journal, Vol. I. nos. 8 and 9, p. 355).

The enzyme is destroyed by heating to 100° C., it seems inhibited at temperatures above 60° C. and most active between 40° C. and 50° C. The cell-proteins with which it is intimately associated appear to shield it somewhat from the otherwise destructive effects of heat.

It seems inhibited or partly destroyed by treatment with alcohol, for an aqueous extract of alcohol-precipitated juice has very little reducing-power compared with the original raw juice.

Drying the juice in vacuo at room-temperature also seems to inhibit its activity: the aqueous extract of juice dried in vacuo does reduce pigments but with greatly diminished vigour compared with the raw juice.

The enzyme is carried down with the cell-proteins, for the precipitate, by saturating juice with amm. sulphate, reduces whilst the filtrate does not.

The enzyme can associate itself either with the globulin or the albumin since the precipitate with $MgSO_4$ reduces, as does also the filtrate from this.

It shows very little inclination to pass out of association with these colloids, and I have not yet obtained it in "pure solution." The nearest approach to this is a watery extract of alcohol-hardened liver, which contains protein and both glycogenase and a reductase. Alcohol injures the enzyme less in intact liver than in its press-juice.

Oxidase is simultaneously present and accounts for the restoration of colour in the upper layers of a mixture of reduced pigment plus tissue-juice even under one inch of toluol.

Keeping the juice in contact with soluble Prussian blue under toluol and an atmosphere of pure Hydrogen for 3 months prevented any trace of surface re-blueing, whereas the colour was at once restored on exposure to the air or by treating the contents of the broken-open tube with H_2O_2.

I have some evidence that the reductase is more "soluble" in glycerine than in any other menstruum.

There appears to be no kinase.

The liver of the frog also possesses a reductase.

The reducing power of fresh kidney-juice is markedly greater than that of fresh liver-juice.

Note on the electrocardiogram, frequency of heart-beat and respiratory exchange in reptiles. By F. BUCHANAN.

In a communication to the Society in March, I suggested that some light might be thrown upon the question as to the sources of the two electric fields created by the beating heart in birds and mammals by seeing whether they were, or were not, present in certain reptiles. I have now found them to be present in the crocodile, the lizard and the snake, and in all these to bear the same relation to one another, both with regard to strength and succession in time, as in mammals, and therefore a different relation from what they bear to one another in birds. We have therefore to seek for the sources of the two fields which arise at the very beginning of the electrical counterpart of the ventricular systole, in some muscular structures which in reptiles and mammals would be similar to one another and which in birds would be dissimilar. Since the two fields exist in the snake and the lizard as much as in the crocodile it cannot be the two ventricles, as such, which serve as the sources.

The most striking difference between the heart of the mammal and the reptile on the one hand, and the bird on the other, lies in the structure of the right auricular-ventricular valve. This in the mammal and reptile is, like the left one, membranous, the flaps near their free edges being attached by chordae tendinae (not described in reptiles) to the papillary muscles in the walls of the ventricle, while in the bird the valve itself is muscular and there are no papillary muscles in the right

ventricle. We unfortunately know very little about the arrangement of the papillary muscles and about their relation to the other musculature (auricular and ventricular) in reptiles, but for mammals we know that they are the only muscles of the ventricle which are connected (by means of the His bundle) with the muscles of the auricle, so that they would be the first of the ventricular muscles to be excited, so long as there were no nerve interference with the order of things. That they should be put into a state of excitation, *i.e.* become tense, before the beginning of the contraction of the walls of the ventricle would serve in the period of increasing tension to prevent the flaps from being inverted into the auricle. Pending further information as to the relation of the muscles of the ventricles to those of the auricles in reptiles and birds, I would now suggest that it is the two sets of papillary muscles, one to each auricular-ventricular valve, which are the sources of the two nearly equal fields created at the onset of the electrical counterpart of the systole in the mammal (and reptile), and that they, by not beginning to act at precisely the same moment, those of the right valve normally beginning some 0·001—0·01 sec. before those of the left, give rise to the effect which Einthoven calls *R* in the electrocardiogram. The effect *T*, which is similar in direction in birds to what it is in reptiles and mammals, I take to be a conus effect, that produced by the aortic conus usually outlasting that produced by the pulmonary conus, the want of effect, or the very small effect, in the intervening period signifying that all the muscles of the ventricle or ventricles are in a nearly equal state of excitation during the time represented by it. So far as reptiles with a one-ventricle heart are concerned, a want of synchronism between the closure of the two auricular-ventricular valves and between the expulsion of the oxygenated blood through the right systemic arch and of the deoxygenated blood through the pulmonary arch, seems to me to be essential for the efficient working of the heart.

Records taken on six different occasions with a young crocodile (*C. americanus*) show that the frequency of the heart-beat varied between 22 and 47 per minute, those taken with two lizards (*L. viridis*) show that in each it varied between 60 and 80 per minute, and those taken with a snake (*Coluber natrix*) show a variation in frequency of from 23—40. Even on the same day and therefore at the same temperature the frequency varied from time to time, whenever the animal became restless the higher frequencies being observed. In all three classes of reptiles the systole is very long, and longer than the diastole. Its duration varies with the frequency quite as much as that

of the diastole does. The following table shows the relative durations of the two:

	Weight in grms.	Frequency per minute	Duration of systole	Duration of diastole
Crocodile	71	22	1·5 sec.	1·2 sec.
		41	0·8	0·65
		47	0·76	0·5
Lizard A	19	60	0·6	0·4
		66	0·5	0·4
„ B	15	63	0·5	0·45
Snake	169	23	1·5	1·1
		41	1·0	0·45

Dr Haldane kindly determined the respiratory exchange in the crocodile at 20° C. The results, reduced to standard pressure and temperature, give:

0·061 grm. per kilo per hour as the CO_2 output,
0·052 „ „ „ „ „ „ „ O_2 intake.

The expenses of the work have been defrayed out of a grant from the Royal Society.

Action of the Nux Vomica Alkaloids (Strychnine and Brucine) upon Muscle. By V. H. Veley and A. D. Waller.

(*Adjourned till Jan.* 22.)

So far as chemical investigations have gone both these alkaloids contain a hydropyridine and a quinoline residue[1], brucine being dimethoxystrychnine. Their constitutional formulae are doubtful.

As regards their affinity it has been established by various methods that brucine is much the stronger[2] (Strychnine $Kb = 1{\cdot}42{\cdot}10^{-7}$
Brucine $Kb = 7{\cdot}20{\cdot}10^{-4}$).

As to their physiological activity it is stated in text books on pharmacology that brucine is 30 to 40 times weaker than strychnine. Lauder Brunton[3] expressed the view that the difference was one of degree rather than of kind, and dependent upon the more ready elimination of brucine.

Our experiments were made on immersed muscle, as previously described, with solutions of the hydrochlorides of the alkaloids, which

[1] Hanssc. *Ber.* xx. p. 451. 1886.
[2] Berthelot and Gaudechon. *Comptes-rendus*, cxl. p. 715. 1905. Veley. *Trans. Chem. Soc.* xcv. pp. 764–5. 1909.
[3] Lauder Brunton. *Journ. Chem. Soc.* (*Trans.*) p. 143. 1885.

were quite neutral in their reaction towards methyl-orange and not hydrolysed in solution. They show that activity, as measured by rate of abolition of muscular contraction, is greater (about 3 times) in the case of strychnine than of brucine, molecule for molecule, and that the permanent effect of the latter is greater than that of the former: the recovery from strychnine is more complete and regular than that from brucine, signifying that the chemical action between the poison and the muscle is more fixed or more profound in the case of brucine than that of strychnine, although in the latter case it is the more intense.

Experiments of June 10th to 12th (Temp. = 18°) 1909.

				Time of abolition of muscular contraction	
378	Strychnine HCl	*n*/400	0·08 %	13 minutes.	
379	Brucine HCl	*n*/200	0·2	32 ,,	
380	Strychnine HCl	*n*/200	0·17	14 ,,	
381	Brucine HCl	*n*/200	0·2	25 ,,	
382	Brucine HCl	*n*/200	0·2	34 ,,	See Figs. 1 and 2.
383	Strychnine HCl	*n*/200	0·17	14 ,,	
384	Strychnine HCl	*n*/800	0·04	60 ,,	(Incomplete abolition.)
385	Brucine HCl	*n*/200	0·2	38 ,,	

Comparing our present results with those previously obtained with quinine and aconitine under similar conditions, we find that strychnine is of considerably lower toxicity in relation to muscle than either of these drugs. Either a *n*/10000 (0·006 %) solution of aconitine hydrochloride, or a *n*/1000 (0·03 %) solution of quinine hydrochloride, is more active upon muscle than a *n*/200 (0·17 %) solution of strychnine hydrochloride. We give provisionally the following numerical estimate of the relative molecular toxicity of these drugs as regards muscle:—

Aconitine = 1000
Quinine = 100
Strychnine = 12
Brucine = 4

The comparative activity on muscle of quinine and strychnine is 100 to 12. Their pharmacodynamic activity on the human subject as indicated by their official doses (quinine 1 to 10 grains, strychnine $\frac{1}{60}$th to $\frac{1}{15}$th grain) in the reverse order, viz. strychnine to quinine as $\frac{60}{1}$ to $\frac{15}{1}$. We call attention to this point in order to make it quite clear that we are dealing with effects of drugs on muscle without drawing inferences as to their relative activity on the organism.

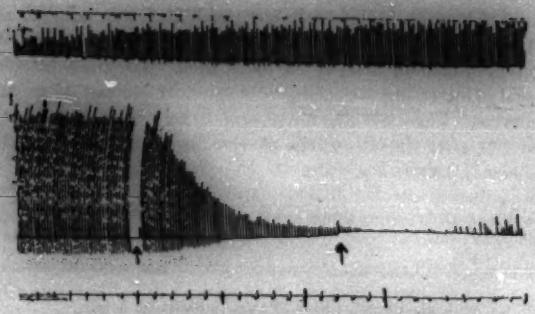

Fig. 1. Strychnine hydrochloride (neutral) n/200 (0·17 %).

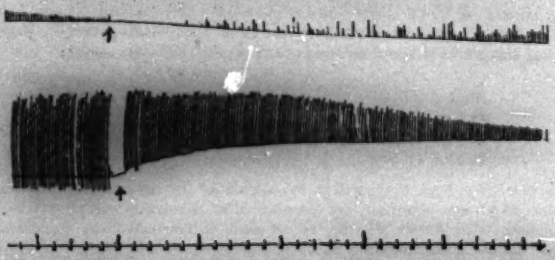

Fig. 2. Brucine hydrochloride. n/200 (0·2 %). In each of these records the immersion in the toxic solution commences at the interval opposite the first arrow ↓; immersion in saline commences opposite the second arrow, and the degree to which recovery takes place is shown by the upper line of each record.

Experiments on Lactic Acid Formation in Man. By J. H. RYFFEL.

(*Adjourned till Jan.* 22.)

Violent Muscular Exercise. The following experiments show that by the use of my method (This *Journal*, XXXIX. p. ix) a considerable increase of lactic acid may be demonstrated in the urine passed after a short period of sufficiently violent muscular exercise, and a corresponding but smaller increase in the blood. Although excess of lactic acid disappears from the urine in about 30′ from the end of the period of exercise, that in the blood has not quite disappeared in 45′. The

acidity of the urine is definitely increased, but the mineral acidity (Folin) is negative, showing that lactic acid excretion leads to an elimination of mineral bases at the same time. The effect therefore of lactic acid in increasing the acidity of blood should outlast its appearance in the urine. These results correspond closely with the changes in alveolar CO_2 after violent exercise observed by Douglas and Haldane (This *Journal*, XXXVIII. p. 433).

(4. IX. 09.) J. H. R. Urine (80 c.c. in 47') passed 30' after running 10 laps (33 laps to the mile) in 2', until thoroughly dyspnœic, contained 454 mg. lactic acid. The acidity was $=23{\cdot}8$ c.c. $\frac{N}{10}$ NaOH per hour. Urine passed 1 hr. 45' later contained 6·42 mg. lactic acid $=3{\cdot}67$ mg. per hour. Its acidity was $=11{\cdot}4$ c.c. $\frac{N}{10}$ NaOH per hour.

Normal day urine contains 3·5—4 mg. lactic acid per hour.

(10. IX. 09.) J. H. R. Urine 155 c.c. passed 17' after running 12 laps in 2' 45" contained 362 mg. lactic acid. 33·5 c.c. passed 1 hour later contained 68·5 mg. lactic acid. Blood was drawn from a vein of the forearm and gave the following results:

Blood	Lactic acid in mg. per 100 c.c.
19·5 c.c. before starting	12·5
19·5 c.c. immediately after stopping	70·8
18·5 c.c. 45' later	15·9

The blood was freed from protein by coagulation by heating with 100 c.c. ·5 % potassium dihydrogen phosphate, the coagulum being thoroughly washed with the same hot solution. The filtrate was neutralised with sodium carbonate, evaporated and employed for the determination of lactic acid.

(10. XI. 09.) J. H. R. Urine (56 c.c. in 50') passed before starting gave acidity and ammonia per hour equivalent to 13·3 and 11·7 c.c. $\frac{N}{10}$ NaOH respectively. Urine (158 c.c. in 44') passed 32' after running 20 laps in 5' 5" with much dyspnœa gave acidity and ammonia per hour equivalent to 38·4 and 28·9 c.c. $\frac{N}{10}$ NaOH respectively.

The latter urine contained 818 mg. lactic acid and gave the following results:

In 100 c.c. total acidity	17·84 c.c. $\frac{N}{10}$ NaOH	
,, ,, ammonia	13·44	,,
,, ,, mineral acidity (Folin)	−44·88	,,
,, ,, total organic acidity	62·72	,,
,, ,, 518 mg. lactic acid	=57·6	,,

As the total organic acidity partly includes CO_2, lactic acid is clearly the only organic acid present in any considerable quantity.

Urine 78 c.c. passed 37' later contained 8·23 mg. lactic acid.

Continued Exercise (17. IX. 09). The urines of competitors in the Blackheath Harriers' 24 hours track walking race were estimated for

lactic acid, but in no case, whether early in the race, or at the finish, was the elimination of lactic acid greater than 6·5 mg. per hour. The average was about 4 mg. per hour. The urines however contained acetone, and in some cases in the final period albumen.

High External Temperature (15. IX. 09). The sweat collected during 35′ in the hot room of a Turkish bath (205° F.) contained 80·6 mg. lactic acid and readily gave the thiophene test. At the end of the period the rectal temperature was 102° F. Pulse, standing, 140 per minute. Urine (192 c.c. in 1 hr. 42 min.) gave 3·54 mg. lactic acid per hour, which is about the normal figure.

Shortage of Oxygen. At no stage in the following experiments did the urine contain more than the normal 4 mg. lactic acid per hour, nor was the acidity appreciably increased. In the first three experiments apparatus was employed similar to that used by Haldane and Poulton (This *Journal*, XXXVII. p. 390). Samples of alveolar air were taken at the end of each experiment. The pulse was counted during the last half minute.

	Subject	Duration of Exp.	Alveolar Air CO_2 %	Alveolar Air O_2 %	Pulse per minute
(13. x. 09)	J. H. R.	10′	4·9	5·35	120
(18. x. 09)	,,	8′ (very dyspnœic)	3·02	3·95	120
(2. xi. 09)*	,,	15′	4·27	4·00	—

The normal rest pulse of J. H. R. is 80 per minute.

* During the last minute of this experiment blood (13 c.c.) was taken from a vein of the forearm. This contained 15·5 mg. lactic acid per 100 c.c.

(27. xi. 09.) J. H. R. 3 hrs. 22 mins. were spent in a small respiration chamber without ventilation, the carbon dioxide being absorbed by soda lime. Reduction of the oxygen was assisted by the use of 2 oz. pyrogallic acid.

Time from start Hrs.	Mins.	Air of chamber CO_2 %	Air of chamber O_2 %	Alveolar Air CO_2 %	Alveolar Air O_2 %	Pulse per minute
2	5	1·3	11·98	5·09	7·43	88
3	0	1·54	8·49	4·69	4·69	96
3	10	1·87	7·78	4·23	4·15	120

The oxygen of the chamber was low enough to reduce alveolar CO_2 for over an hour, the normal alveolar CO_2 of J. H. R. being about 5·3 %.

Increased respiration, cyanosis and mental confusion were observed. Headache became marked after the admission of fresh air at the end of the experiment.

Urine passed at the end of the experiment gave definite tests for acetone and oxybutyric acid, but contained no other abnormal constituent.

Dr Pembrey kindly analysed the above samples of air.

(16. xi. 09.) Through the kindness of the Director an experiment

was made at the Lister Institute in the pressure chamber presented by Dr Ludwig Mond. My thanks are due to Dr Boycott, who made the necessary arrangements and took part in the experiment. This lasted 4 hours 10 minutes at a pressure of 450—460 mm. Hg. with adequate ventilation. The pressure was sufficiently low to reduce the alveolar CO_2 appreciably (Boycott and Haldane, This *Journal*, XXXVII. p. 390). The temperature varied from 12·5° to 18° C.

Subject	Time from start Hrs.	Mins.	Alveolar CO_2 in mm. Hg.	Pulse per minute	Blood-pressure in mm. Hg.
A. E. B.	0	45	39·8	—	—
,,	2	25	—	72	96
,,	3	35	37·2	—	—
,,	4	0	—	78	104
J. H. R.	0	30	—	90	—
,,	1	0	33·7*	—	—
,,	2	25	—	99	135
,,	3	50	33·3	122	157

* After inspiration only.

Lunch was taken after 2 hrs. 35 mins. in the chamber.

Before the start A. E. B.'s alveolar CO_2 was 45·6 mm. His usual normal alveolar CO_2 is 42 mm., pulse 75 per minute, blood-pressure 100 mm. Hg.

J. H. R.'s alveolar CO_2 normal is 40·5 mm., pulse 80 per minute, blood-pressure 120 mm. Hg.

Towards the end of the experiment A. E. B. was cyanosed and suffered from violent headache and nausea, but J. H. R., though cyanosed, had only slight headache. The symptoms of both were of gradual development. The urine of A. E. B. contained no abnormal constituent, that of J. H. R. a trace of acetone. Blood (15 c.c.) was taken from a vein of the forearm of J. H. R. just before the end of the experiment. This contained 23·6 mg. lactic acid per 100 c.c., an increase from the normal equivalent to 2·7 c.c. CO_2 per 100 c.c., assuming a molecule of lactic acid = a molecule of CO_2.

The oxygen in inspired air at a pressure of 450 mm. is equivalent to 11·8 % at 760 mm. Assuming a respiratory quotient of ·9, A. E. B. had in his alveolar air at the end of the period 43 mm. = 6·02 % O_2. J. H. R. had 47·2 mm. = 6·62 % O_2.

The above results show that in each case the shortage of oxygen was sufficient to reduce the alveolar CO_2, but not sufficient to cause lactic acid to appear in the urine. There was a definite increase of lactic acid in the blood of the forearm after an experiment lasting four hours, but not after one of 15′, during which the oxygen shortage became very marked. It would appear therefore, that in the accumulation of lactic acid in the general circulation, due to lack of oxygen, time is an important factor. As to lactic acid in the respiratory centre, or in the jugular vein, no conclusions can be drawn.

The effect of oxygen inhalation on muscular exertion. By LEONARD HILL and J. MACKENZIE.

IN the Proceedings of this Society, June 26, 1909, Drs Haldane and Douglas gave the results of some observations on themselves of the effects of running twice up and down a 40 ft. flight of stairs after *forcibly* breathing air for three minutes or *quietly* breathing oxygen, and conclude that "Hill's recent observations as to the effects of previous inhalation of oxygen in diminishing the distress caused by muscular exertion can be explained as a result of the deep breathing which is apt to occur in persons to whom oxygen is administered."

To refute this statement we offer the following evidence.

Taking forcible breathing itself as a type of exertion we find that forcible breathing air, as is well known, becomes very uncomfortable. There is a feeling of constriction round the head and of "pins and needles" in the limbs. Most subjects who try it for the first time say the feeling is like the first stage of going under nitrous oxide gas. If continued there arises lessened force in the breathing and group breathing sometimes of a very marked character, as in one of us who on each of two occasions found the experience so unpleasant that he is unwilling to repeat it.

Tracings show an acceleration and enfeeblement of the pulse during forced breathing, and the pulse can be felt with the fingers to become weak with each inspiratory effort, and return with expiration. Readings of blood pressure show a fall in pressure and especially during each inspiratory effort. In one case the polygraph record showed that the pulse almost ceased to move the lever during the time of three or four beats when the forced breathing was temporarily arrested. The heart and respiration apparently were working in rhythm together, and when one stopped the other failed to fill properly.

Forced breathing of oxygen produces at first slight and fleeting unpleasant feelings. The pulse quickly becomes strong and the blood pressure, although oscillating with each inspiration, is maintained. In every case but one (M. G.) there was little evidence of group breathing when oxygen was inhaled, and the forced breathing of oxygen could be continued almost indefinitely without any feeling of effort.

It appears to us that forcible breathing mechanically interferes with the circulation and the aeration of the heart and brain. The symptoms felt in the head are not unlike those felt on holding the breath.

Breathing through a long tube in the method of Haldane and Douglas must intensify this mechanical effect by increasing the negative pressure in the thorax during each inspiration, owing to the resistance in the tube. They obtained Cheyne Stokes breathing by means of the long tube which they attributed to oxygen want, and removed it by breathing oxygen through the tube. We have obtained the same results, but in most cases in a minor degree, by mere forcible breathing without a tube.

Another method we have used is to test the number of times we can pull up a 60 lb. weight 19 inches high while holding the breath before the "bursting" point, and in what time this occurs, firstly after quietly breathing, secondly forcibly breathing air, thirdly oxygen. Fatigue obviously is against the oxygen results.

	After quiet breathing	After forcibly breathing 3 mins.	After forcibly breathing oxygen 3 mins.
L. H.	12 pulls in 19 secs.	33 pulls in 43 secs.	60 pulls in 70 secs.
	13 ,, 18 ,,	—	—
R. A. R.	33 ,, 60 ,,	—	—
	(Gave two snorts at 45 secs.) Very exhausted and cyanosed, on second attempt with nose clip on could only do		
	11 pulls in 17 secs.	34 pulls in 45 secs.	49 pulls in 70 secs.
M. F.	17 ,, 26 ,,	59 ,, 73 ,,	69 ,, 78 ,,
	23 ,, 30 ,,	—	—
J. M.	17 ,, 23 ,,	30 pulls in 50 secs. (Marked Cheyne Stokes during deep breathing, audible to every one in room, unconscious of them himself)	75 pulls in 85 secs. (No Cheyne Stokes during inhalation)
M. G.	29 pulls in 29 secs. Very done out	43 pulls in 54 secs. Very done out	49 pulls in 68 secs. Stopped in good state

Each one remarked the ease with which he pulled after oxygen.

None of us after forcibly breathing air have been able to hold our breath while sitting quiet more than 2—3 minutes, but after oxygen 5—7 minutes, and in the case of Greenwood 8 min. 35 sec. on a first attempt and 9 min. 3 sec. on a second attempt. This was after forcibly breathing oxygen for 5 min., and the observation was controlled by a polygraph record of pulse and respiration.

In the above experiments to avoid the influence of the "dead space" of a mask, we have used a wide mouthpiece fitted with mica valves, and to make the conditions the same have breathed in some cases breathed air from the bag as well as oxygen. Hot water has been put in the bag to moisten the oxygen.

Addendum.

The following are the records of two runners (medical men) obtained by one of us (L. H.), giving the effects of deep breathing air and oxygen.

P. T. ('Varsity blue), out of form. Race, 250 yards, unpaced.

Pulse-rate	Time of run		Condition
		AIR.	
68	—		Normal.
148	29½ sec.		Immediately after race. Vomited and had severe gastric disturbance.
		OXYGEN.	30 mins. later.
128	28 secs.		Immediately after race. Pulse very full and strong, and face very good colour. Felt he ran better. Afterwards vomited again.

C. T., half-mile runner of repute, out of form. Race, half-mile, paced last 250 yards.

Pulse-rate	Blood-pressure	Time of run	Condition
		AIR.	
66	125	—	Normal.
165—170	70—80	2.14½	Immediately after. Very much done, pulse weak and irregular, difficult to count. Wants to bend down.
146	110	—	One minute later.
		OXYGEN.	30 mins. later.
128	120—5	2.16	Not nearly so done. Holds himself upright. Much better colour. Pulse notably fuller and stronger. Says his legs gave out. Feels fit to run again.
132	—	—	A minute later.

C. T. A week later. Half-mile, entirely unpaced.

Pulse-rate	Blood-pressure	Time of run	Condition
		OXYGEN.	
148	140—5	2.12½	Immediately after run. Held himself upright; not so done as after first race last week. Pulse regular and strong.
132	—	—	After one minute.
		AIR.	30 mins. later.
160	125	2.21	Immediately after run. Pulse regular. Legs gave out. Not so done as first race last week.
152	—	—	After one minute.

These gentlemen were quite unbiassed, and each time ran themselves out to the utmost. Their times and pulse, feelings and appearance were all in favour of the beneficial effect of oxygen.

The rate of action of drugs on Muscle as a function of temperature. By V. H. VELEY and A. D. WALLER.

(*Adjourned till Jan.* 22.)

We tested the problem by observations on the rate of action on muscle of Alcohol, Chloroform, Quinine and Aconitine at temperatures between 7° and 25°.

We used Esson's formula, modified for our purpose, for the calculation of results

$$\log L_0 - \log L_1 = m (\log T_1 - \log T_0)$$

(where L_0 and L_1 are the lengths of time between application of the drug and cessation of contraction, and T_0 and T_1 the absolute temperatures at which the action took place; m is the experimental constant).

The values of m came out as follows:—

Alcohol = 20·5; Chloroform = 14·3; Quinine = 26·7.

(The values of m in the case of hydrogen peroxide and hydrogen iodide = 20·38, and in that of chloric acid and ferrous sulphate = 26·5.)

The corresponding temperature-coefficients per 10° are:—

Alcohol = 2·02; Chloroform = 1·63; Quinine = 2·52.

(In a previous rough determination we found Ether = 2.)

The data from which the value of m was calculated in the case of chloroform are as follows:—

Temperature	Lengths of time	$\log \frac{T_1}{T_0}$	$\log \frac{L_0}{L_1}$	m
7°	24·5′	0000	0000	—
10°	21′	0047	0670	14·3
19°	13′	0182	2753	15·1
24°	10·5′	0257	3673	14·3
24°	11′	0257	3478	13·5
			Mean =	14·3

The action of aconitine is completely arrested at 7°, and manifests itself as soon as the temperature is raised to 17°.

Our data are described and discussed at length in a paper communicated, November 22, to the Royal Society.